THE EFFECT OF TURBULENCE ON BACTERIAL SUBSTRATE UTILIZATION

A THESIS

Presented to

The Faculty of the Graduate Division

by

John T. Marlar

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Sanitary Engineering

Georgia Institute of Technology

December, 1967

THE EFFECT OF TURBULENCE

ON BACTERIAL SUBSTRATE UTILIZATION

Approved:

Chairman

Date approved by Chairman 3-17-68

ACKNOWLEDGMENTS

Patience, knowledge, and a willingness to work are prerequisite to most worthwhile endeavors.

The writer's wife, Maryjo, supplied much needed patience to the writer when it was needed most. Appreciation is also extended for her understanding during a year which was most trying.

Special thanks are extended to Doctor W. E. Gates for supplying the basic concept and the knowledge with which to complete this research. His willingness to work affected all those who were associated with him during this year.

The writer is indebted to many for their assistance during this research, but none so deeply as the two mentioned above.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

SUMMARY

Traditionally, the processes of deoxygenation and surface reoxygenation occurring after the introduction of an organic waste into a system containing bacteria have been considered to follow first order kinetics. The rate of removal of substrate by bacterial action with concomitant removal of oxygen from the system has been considered to be dependent on *only* the concentration of the remaining substrate.

Since it is reasonable to expect that variations in the initial numbers of bacteria and their physical environment should affect the subsequent interactions between the bacteria and substrate, the approach taken in this investigation utilized a mathematical model capable of separating these effects. The model used is based upon the approach to bacterial growth kinetics as proposed by Monod (1). The basic relationship of the Monod formulation is:

$$k = \frac{k^m X^n}{K + X^n} \qquad (1)$$

The approach as advanced by Monod takes issue with the traditional approach in that it provides a mathematical basis for the relationship between bacterial growth and the resulting disappearance of substrate.

The interaction of a heterogeneous population of bacteria with a simple well defined substrate (glucose) was studied using a reactor in which the predominant reactions occurring in nature could be observed.

The processes of bacterial growth, substrate removal, deoxygenation and surface reoxygenation could be studied under controlled environmental conditions. The effect of turbulence upon bacterial substrate utilization was demonstrated by changing the turbulence level in successive tests. The disappearance of the substrate as it was consumed by a heterogeneous bacterial population was determined in each test.

The experimental system consisted of a complete mix batch reactor with mixing provided by an impeller driven with a variable speed electric motor as the prime mover. The processes of deoxygenation and reaeration were monitored continuously by utilizing a Galvanic Cell Oxygen Analyzer (D.O. probe) in conjunction with a recorder. An enzymatic test specific for glucose permitted monitoring of substrate removal. Bacterial activity was determined using a test for the dehydrogenase enzyme as developed for this study.

Based upon the system studied, the following conclusions are made:

(1) The bacterial growth kinetic approach presented can be utilized quite well with a heterogeneous bacterial population.

(2) The effect of varying initial bacterial concentrations on the system is important and can be predicted.

(3) Measurement of viable biomass in dilute systems is possible using a modified dehydrogenase test.

(4) The oxygen utilization by the bacterial population is directly proportional to the utilization of substrate.

(5) The utilization of substrate did not follow first order kinetics, but followed the form as predicted by the mathematical model

presented by Monod (1).

(6) The maximum bacterial growth rate was a constant.

(7) Environmental conditions affecting the growth rate of the bacteria and the rate of substrate removal were reflected in the value of the substrate saturation constant, K.

(8) Increasing the level of turbulence in the bacterial-substrate system increased the bacterial growth rate and the rate of substrate removal.

CHAPTER I

INTRODUCTION

General Consideration of the Problem

Stream analysis in the past has been concerned with evaluating the oxygen resource of streams subject to organic pollution. The oxygen resource of a stream is renewable; with the dissolved oxygen concentration existing at a point in a stream representing the net cumulative effect of the preceding oxygen sources and sinks. The biological and/or chemical stabilization of organic pollutants represents the most significant oxygen sink of a stream. The total oxygen so consumed represents the oxygen equivalent of the pollutant. The major uninterrupted source of oxygen to the stream is from the atmosphere through surface reoxygenation, although tributary streams and algal activity can supply significant amounts of oxygen in specific situations.

An oxygen sag curve is the result of two opposing reactions, that is, the utilization of oxygen in biological and/or chemical stabilization of organic pollutants and the transfer of oxygen from the atmosphere to the stream. An oxygen sag curve normally results only if initially the rate of deoxygenation exceeds the rate of reoxygenation and if at some point later in time the situation is reversed. These necessary interrelationships can be a consequence of the metabolic activities of microorganisms as they consume an organic pollutant. The rate of oxygen utilization is highest when the organisms are actively growing at the

expense of the pollutant and decreases after the pollutant is essentially depleted.

The use of oxygen analyses to indirectly determine the rate of disappearance of organic pollutants is desirable since it is possible for oxygen analyses to be accurate and simple and to relate directly to the resource being considered. The development of selective membrane electrodes and equipment suitable for continuously monitoring oxygen levels has further enhanced the desirability of this approach.

The prediction of future stream conditions using deterministic relationships has been subject to considerable study in the past. The adequacy of such predictor systems is dependent on the degree to which the effects of deoxygenation and reoxygenation can be separated and identified.

Kinetic Models

There is general agreement that the process of surface reoxygenation is a true first order reaction whose velocity is controlled by the driving force (oxygen deficit below some saturation value) and the resistance to transferring oxygen from the atmosphere to the hydrosphere (2).

The kinetics of deoxygenation have been less clear and general agreement has not been realized. The lack of concensus is primarily due to a lack of differentiation between bacterial growth and death in conjunction with the lack of adequate testing techniques to describe complex wastewaters in terms of parameters suitable for rigorous mathematical treatment.

In an attempt to describe the conditions existing in the Ohio River, Streeter and Phelps (3) presented an equation describing both the processes of reoxygenation and deoxygenation in terms of first order reactions. These investigators wisely warned that the equation presented not be used indiscriminately. After examining many BOD tests performed with Ohio River and other waters, Streeter and Phelps represented the process of deoxygenation as following first order kinetics. The performance of the BOD test and the use of the results in the oxygen sag equation therefore assumes that the bacterial utilization of oxygen is basically the same whether it takes place in a bottle under somewhat controlled conditions or in a free flowing stream.

There are several operational difficulties which have hampered investigators using this first order approach. All the parameters in the equation are measured or estimated, using combinations of field and laboratory data, leaving only one unknown, the rate of reoxygenation k_2, which is then determined from the equation. The applicability of the oxygen sag equation is then verified by its ability to fit the oxygen data obtained from field measurements. This procedure, of course, is highly subjective in nature. However, the Streeter-Phelps sag equation is often employed as a fundamental expression to which field data must conform. Deviations from the model are usually attributed to unknown causes, or, as Fair (4) pointed out, to differences in rates of reaeration when differing rates of reaeration are only one of the many variants in the prediction of stream behavior.

Monod (1) presented an expression which is especially pertinent to stream analysis. It describes the growth of organisms as they

utilize a substrate as an energy source. The development of the Monod equations describing the growth of organisms and the disappearance of substrate in batch culture is presented in Appendix 1.

Monod's investigations showed that his kinetic model could be used to describe the growth of pure cultures on several well defined simple substrates.

Possible Methods of Approach

Many investigators have chosen to study the "self-purification" of streams in nature (5,6,7). Studies such as these can often lead to the solution of an immediate problem, but do little to identify the individual effects of any of the potential variants. The fallacy of this approach is that any solution obtained may be unique to the situation studied.

A more logical method of attack, often applied to complex problems, is to construct a less complex system with a minimum of known variables. In this manner, the effects of selected variables under controlled conditions can be studied. The system employed can be made increasingly more complex as the predominant variables are identified and evaluated. It is apparent that this approach has the potential to produce meaningful insight into the effects of selected variables.

Method of Approach Selected

In an application of a laboratory model to study the assimilative capacities of streams, Mancy and Gates (8) considered it necessary to revert to a "building block" approach in order to obtain meaningful data pertinent to stream analysis. The approach is based upon the use

of a simplified system simulating the basic reactions occurring in nature, that is, surface reoxygenation and deoxygenation. An environment should be provided such that temperature, rate of reoxygenation and therefore turbulence level, substrate, and initial organism concentration are controlled. Ideally, all parameters except the one of interest should be held constant while a parameter of interest is varied. These investigators considered the measurement of k_2, that is, $k_L a$, *in situ* essential if the results of simulation studies were to be applied to stream analysis. It is evident that the value of $k_L a$ represents a common parameter of comparison between streams since it can be considered dependent only upon the physical characteristics of the stream.

In an earlier work using the approach described above, Gates et al. (9) concluded that the Monod expressions appeared to be flexible enough to represent the deoxygenation process, but did not appear to be desirable from an operational point of view. It was suggested that consideration be given to second order and autocatalytic reactions to determine the conditions under which they represent acceptable approximations of the Monod equations as applied to batch cultures. However, these approximations are not necessary when the analytical techniques as presented by Gates and Marlar (10) are used. Also, such approximations are particularly undesirable if the effects of system variables are to be accurately determined.

The method of approach selected for the present investigation is based upon the earlier work of the two investigations previously mentioned. A simplified system using controlled environmental conditions

was constructed and used to determine the effects of the variables selected for study.

Purpose of the Study

From the preceding discussion, it is evident that a demonstration of the applicability of the Monod equations to batch cultures and the effect of varying levels of turbulence represent a logical first step in the ultimate simulation of a polluted stream using a laboratory model. The purpose of the present investigation then is to extend the initial efforts of Gates et al. (9) and Mancy and Gates (8) by:

(1) demonstrating the applicability of the Monod nutrient equation to a batch system using a defined substrate and a heterogeneous bacterial population, and

(2) demonstrating the effect which one variable, turbulence as measured by $k_L a$, has on bacterial substrate utilization.

CHAPTER II

LITERATURE REVIEW

General

This literature review is a pertinent but not exhaustive review of the techniques used and observations made by other investigators concerned with bacterial substrate utilization. The literature reviewed is not only from the sanitary engineering field, but from related fields as well. Sanitary engineers have too often, in the past, ignored the information available concerning bacterial growth kinetics and other pertinent observations made by the microbiologist.

Where appropriate, results reported in the literature are interpreted in light of the Monod model.

The Monod Model

Monod (1) presented a quantitative description of the growth of microbial populations based on the concept of a growth limiting parameter. In Monod's initial work, the carbon source, or energy source, was growth limiting. It was demonstrated that the kinetic model developed could adequately describe the growth of several species of pure cultures on many defined substrates. Monod (11) later pointed out that the kinetic expression presented is empirical in nature, but is convenient and logical to adopt. The mathematical form of the expression is the same as the Michealis-Menten equation used in the field of enzyme kinetics and is a reasonable functional expression of the Law of the

Minimum. The former has been accepted as an adequate theoretical and practical expression to describe the behavior of enzyme-substrate systems (12,13). The derivation of the Monod expression applying arguments similar to those which result in the Michealis-Menten relationship to a bacterial-substrate system is included in the appendices. The question as to whether the Monod expression applies without equivocation to bacterial-substrate systems with the same meaning as the Michealis-Menten relationship to enzyme kinetics cannot be ascertained at this time. The use of the Monod equations as a "process kinetic" tool, however, has received wide acceptance (9,15,16,18,19).

To date, the major application of the Monod equations has been in studies using complete mix continuous flow reactors (8,14,15,16,17). The resulting mathematical expressions are quite simple and easily solved when applied to complete mix continuous flow systems since steady state conditions can be achieved. A thorough discussion of continuous flow culturing techniques is presented by Malek (18). The evaluation of the constants of the Monod equations is relatively simple once data is obtained from continuous flow systems, but the time involved in data procurement is often lengthy. Conversely, the evaluation of kinetic constants from the Monod equations as applied to batch systems has been quite involved while the data is easily obtainable.

Several investigators (19,20) have employed computers as an aid for evaluating the constants in the Monod equations as applied to batch systems. Values of the bacterial yield factor were assumed, based on reported values, and changes in a specific substrate concentration were measured as a function of time. The remaining constants were determined

through the use of a computer oriented non-linear least squares technique for obtaining the "best fit" of the data. Gaudy et al. (15) obtained an approximate solution by measuring the increase in dry weight solids and presumably the decrease in substrate concentration with time in a batch system. Using this technique, the average growth rate for a time interval was calculated. By plotting the growth rates obtained against the average substrate concentration measured during the time interval, these investigators were able to determine approximate values of the required constants. This technique is essentially that used by Monod (1). As pointed out earlier, Gates et al. (9) have shown that the Monod equations definitely demonstrate the flexibility required to describe the data obtained from batch cultures, but concluded that simplifications of the equations might be necessary in order to apply them to batch systems. Recently, however, Gates and Marlar (10) presented a procedure for transforming the batch equation to a linear form which permits rapid determination of the various rate constants with a minimum of effort. This technique was used in the analysis of data obtained in this investigation. Wider use of this technique should greatly enhance the use of the Monod approach to bacterial growth kinetics.

The applicability of the approach adapted from enzyme kinetics and applied to bacterial growth kinetics by Monod is becoming increasingly apparent as evidenced in the literature. The pertinence of this approach to biological systems has been recognized for some time as pointed out by Fair and Geyer (21) and Rich (22) among others.

Similar Investigations

The approach used in this investigation has been used by other investigators to study the utilization of substrate by a dilute bacterial system.

Schafie (23) used a small complete mix batch reactor, a pure culture of *Escherichia coli,* and several defined pure substrates to observe their interaction. The substrates used, added both singly and in combination, consisted of glucose, acetate, lactose, glucose and lactose, and glucose and acetate. Some data obtained using raw sewage and Ohio River water were also presented. Schafie concluded that the results of his studies indicated that the bacterial oxygen uptake conformed to the classical first order BOD equation. Analysis of data taken from his work indicates that first order kinetics are only a very rough approximation and that the Monod equation represented the data quite well. This observation is also confirmed by Gates et al. (9) in an extension of the approach taken by Schafie. These investigators found that the use of first order kinetics could not explain the data obtained and concluded that for the system studied, the monomolecular expression for oxygen uptake by the biosphere was invalid. The effects of sequential substrate utilization and predation as parameters which affect the shape of the oxygen sag curve were also considered.

Isaacs and Gaudy (24) used a rather unique device to simulate a flowing stream under laboratory conditions in order to study the equivalence of the BOD test to comparable data taken from a turbulent system. The device consisted of a tank in the shape of a right circular cylindrical torus. Provision was made to control the velocity of the

water by the use of movable inner walls. Bacterial organisms from sewage were used with glucose, a mixture of glutamic acid and glucose, and diluted sewage as substrates. The fate of the substrate was followed as well as the resulting D.O. sag curve. The results obtained very closely resemble the results presented later in this thesis. Isaacs and Gaudy pointed out that first order kinetics could not be used to explain the data and attempted to use a combination of expressions to reproduce the sag curves obtained. After the initial portion of the data was neglected, a first order equation was used to describe the sag curve to the critical point, followed by two zero order expressions with different rates to represent the data after reoxygenation had begun to dominate the overall process.

Some Possible Variables

Measurement of Bacterial Activity. In the study of bacterial growth kinetics and the interaction of bacteria with various substrates, the techniques employed for measuring the quantities of organisms involved have been many and varied. Both microbiologists and sanitary engineers have used the increase in optical density (1,15) as a measure of organism concentration during the growth phase. Optical density is either calibrated against the increase in dry weight of bacteria in the log growth phase or used independently as a qualitative measure only. However, this technique is difficult to apply when dilute suspensions of bacteria are used.

Since membrane filters and workable techniques for their application have been developed, the increase in dry weight of suspended

material has often been used (25). Use of membrane filters to determine dry weights requires that the laboratory technique used be followed in exactly the same manner for each measurement. Membrane filters are known to be hygroscopic and great care is necessary to obtain accurate measurements. Time sequence dry weight determinations of growing cultures define the amount of growth which has taken place, but can reveal little about the concentrations of *viable* organisms.

A technique used to indicate the numbers of viable bacteria in a system is the total plate count. The total plate count has long been used in research work (26), but has found little use in mathematical expressions for growth kinetics since such expressions are in some manner related to mass.

Most kinetic expressions require that the measure of biomass be expressed in terms of weight which ideally should represent only the viable biomass in the system (14). Agardy et al. (28) pointed out that kinetic studies are often hampered by the difficulty in measuring the "active" organism concentrations in biological systems. These investigators attempted to develop a method for enzyme activity measurement to allow the condition of the system to be determined. Although the system studied was an anaerobic digester, the point to be noted is that an attempt was made to relate the effect of "active" organisms to the performance of a biological system. Lenhard et al. (29) presented a procedure to determine dehydrogenase activity and used it to measure the activity of organisms in a laboratory activated sludge system. Although not explicitly stated by these investigators, the results obtained can be interpreted as being a measure of the viable biomass in the system.

Ford and Eckenfelder (30) also obtained comparable results in a separate investigation. Hernandez (31) modified the procedure of Lenhard et al. (29) slightly, but presented too few results to permit an evaluation of the modifications. In view of the potential of dehydrogenase activity measurements to be related to viable organisms concentrations, the test was modified for use in dilute bacterial systems and used in the present investigation. The procedure and modifications developed are presented subsequently.

Bacterial Yield. The determination of bacterial yield using a glucose substrate is well documented in the literature. Yield is usually expressed in terms of dry weight of cell material produced per unit of substrate consumed. Quite often the procedure for determining the dry weight of cell material produced is neither mentioned nor adequately detailed in the literature. Thus, it is not known to what extent the differences in reported yield values simply reflect differences in weight determinations. The units of measure of substrate utilized used by the various investigators were quite varied. Some of the units noted were COD, TOD, 5-day BOD, ultimate BOD, and more simply, the weight of the specific substrate utilized.

In a discussion of Monod's work, Pipes and Micholits (32) stated that the yield of a pure culture on a defined substrate should be a constant if the substrate is the sole carbon source and is the growth limiting factor in the system. They also pointed out that the yield should be measured during either log or stationary growth. Moreover, if it is accurately determined, the bacterial yield should be a measure of the energy available from the substrate for growth. Similarly,

Gaudy and Gaudy (33) pointed out that the yield factor for heterogeneous populations is "by no means" a true constant. It should be noted that this statement was contained in a general discussion and was unsupported by data. More recently, however, Gaudy et al. (15) have presented data to substantiate this concept. Unfortunately, the data presented do not substantiate the concept of a variable yield for heterogeneous cultures, but rather reinforced the concept of a constant value for bacterial yield.

The Monod kinetic expressions require the units of the yield factor to be compatible with those used to define the substrate. For this reason, yield values reported in the literature have been recomputed when necessary to compare all values on an equable basis. For this purpose, yield is expressed as dry weight of bacteria produced per unit weight of substrate utilized. When the reference cited provided the unit cell mass produced as COD, a factor of 1.42 mg COD/mg bacterial weight (25) was used. A factor of 1.07 mg COD (or TOD)/mg glucose was also applied when appropriate. A compilation of these data are presented in Table 1. It is believed that all the data listed were obtained from batch systems in which the carbon source, glucose, was the growth limiting factor. It is apparent that these data are in reasonable agreement. In fact, the agreement is such that, for most purposes, the yield factor may be taken as a constant.

Table 1. Bacterial Growth Yields Using Glucose Substrate

Organism	Yield* Y^o (mg/mg)	Reference Cited
Aerobacter aerogenes	0.55	Watson and Hoffee (34)
Salmonella typhimurium	0.51	Watson and Hoffee (34)
Aerobacter globiforms	0.475	Morris (35)
Eschericia coli	0.45	Morris (35)
Mixed Culture	0.37	Servizi and Bogan (36)
Mixed Culture	0.51	Englebrecht and McKinney (37)
Mixed Culture	0.440	Busch et al. (25)
Mixed Culture	0.430	Busch et al. (25)
Mixed Culture	0.433	Busch et al. (25)
Mixed Culture	0.460	Busch et al. (25)
Mixed Culture	0.450	Busch et al. (25)
Mixed Culture	0.453	Busch et al. (25)
Mixed Culture	0.487	Busch et al. (25)
Mixed Culture	0.438	Busch et al. (25)
Mixed Culture	0.455	Busch et al. (25)
Mixed Culture	0.570	Hernandez (31)
Mixed Culture	0.490	Gaudy et al. (15)
Mixed Culture	0.454	Gaudy et al. (15)
Mixed Culture	0.400	Gaudy et al. (15)
Mixed Culture	0.490	Gaudy et al. (15)
Mixed Culture	0.490	Gaudy et al. (15)
Mixed Culture	0.518	Gaudy et al. (15)
Mixed Culture	0.518	Gaudy et al. (15)
Mixed Culture	0.518	Gaudy et al. (15)
Empirical	0.503	McKinney and Symons (38)
Empirical	0.422	Busch, et al. (25)

*Yield is expressed as mg of dry weight bacteria produced per mg of glucose removed.

Growth Rates. The only bacterial growth rate data which can be compared from the literature is the maximum growth rate constant, k^m, determined for non-growth limiting conditions or computed from the Monod equation. Gaudy and Gaudy (33) have indicated that the maximum growth rate of heterogeneous bacterial populations is "by no means" a true constant. The data presented by Gaudy et al. (15) is in agreement with this hypothesis. However, as with the value of bacterial yield mentioned earlier, the data presented does not appear to be conclusive. Maximum growth rate values reported by these and other investigators are presented in Table 2.

Table 2. Maximum Bacterial Growth Rates (k^m) on Glucose Substrate

Organism	Temp. (°C)	k^m (hr^{-1})	k^m* at 20°C (hr^{-1})	Reference
Aerobacter aerogenes	37	1.20	.370	Neidhardt and Frankel (39)
Aerobacter aerogenes	37	1.10	.339	Watson and Hoffee (34)
Salmonella typhimurium	37	1.13	.348	Watson and Hoffee (34)
Escherichia coli	37	1.30	0.400	Moyed (40)
Escherichia coli	37	1.35	0.416	Monod (1)
Mixed Culture	25±2	0.365	0.258	Gaudy et al. (15)
Mixed Culture	25±2	0.350	0.248	Gaudy et al. (15)
Mixed Culture	25±2	0.545	0.386	Gaudy et al. (15)
Mixed Culture	25±2	0.470	0.332	Gaudy et al. (15)
Mixed Culture	25±2	0.595	0.420	Gaudy et al. (15)
Mixed Culture	25±2	0.470	0.332	Gaudy et al. (15)
Mixed Culture	25±2	0.560	0.396	Gaudy et al. (15)
Average of Reported Values		-	0.353	

*Q_{10} = 2.0 was used.

As indicated in Table 2, the temperature of the experimental system used by Gaudy et al. (15) was reported to vary as much as two degrees around an average value of 25°C. The 2.5 liter reactor was not contained in a constant temperature bath and thus responded to changes in the ambient temperature. The average value of k^m reported by Gaudy et al. (15) was 0.479 hr^{-1} at 25°C. Using a Q_{10} value of 2.0 (1,31) and the temperature variations reported, the average value of the maximum growth rate could have been from 0.416 to 0.550 per hour. It is seen that temperature has a dramatic effect on the value of the maximum growth rate and that no conclusive data were presented with which to determine the variability of the maximum growth rate in aerobic systems using glucose as a substrate.

Oxygen Uptake. Since the amount of oxygen used during substrate removal is of particular interest to sanitary engineers and is relatively easy to measure, it is not surprising to find many articles on the subject and numerous differences in reported values.

Busch and Myrick (41) presented an empirical relationship for the aerobic metabolism of glucose by bacteria to new cell material, carbon dioxide, and water which indicated that the original exogenous glucose would be completely metabolized at 37.5 per cent of the theoretical oxygen demand, TOD. In a subsequent article by Busch et al. (25) which presented a short term oxygen demand test, this figure was illustrated by presenting oxygen uptake data from BOD bottle systems using a glucose-glutamic acid substrate. These data indicated good agreement with the empirically derived figure of 37.5 per cent. The occurrence of a plateau in oxygen uptake was interpreted as the

exhaustion of the original exogenous carbon source. In a paper concerned with factors affecting the existence of such a plateau in the course of bacterial substrate utilization, Gaudy et al. (42) reported that 26 to 51 per cent of the TOD had been exerted at the end of the first phase of oxygen uptake. Again, the first phase of oxygen uptake was interpreted as the utilization of exogenous substrate which in this case was glucose. Examination of these data reveal that values of 32 to 37 per cent of the TOD occurred most often, accounting for 17 of the 27 results presented. In a study of the growth and endogenous phases in the oxidation of glucose, McWhorter and Heukelekian (43) found that the original substrate was depleted at 18 per cent of the TOD, while total substrate removal as measured by COD approached completion at 30 per cent of the TOD. A correlation between the initial seed concentration was also noted. The percentage of the TOD utilized for complete removal of the initial substrate (as measured by COD) decreased to a value of 21 per cent as the initial biomass concentration was increased to 1000 mg/l. No conclusion regarding the apparent correlation was presented. It should be noted, however, that the variations observed are within the range of values reported by Gaudy et al. (42). In a discussion of the work of McWhorter and Heukelekian (43), McKinney and Symons (38) pointed out that their empirically derived expressions indicated an oxygen demand of 33 per cent of the TOD for complete bacterial utilization of glucose. The work of Isaacs and Gaudy (24) considered earlier indicated a discrepancy between the amount of oxygen utilized per unit of substrate in the BOD test and that used in the simulated stream. Similar results were reported by Prokesova (44) using bottles

to contain the ecosystem. One set of oxygen uptake data was obtained with air in contact with the water undergoing biological deoxygenation, while other data were obtained from a bottle system in which no contact with the atmosphere was allowed. Both bottle systems were slightly agitated by rotating them slowly on an inclined turntable device. Prokesova (44) noted, as did Isaacs and Gaudy (24), that the amount of oxygen utilized in the closed system was consistently higher than in the open system. Conversely, Nejedly and Pelz (45) found that reaeration during the course of the BOD test had no effect on the results obtained. No explanation for the differences reported in the literature is readily apparent.

The oxygen plateau value mentioned earlier is often used by researchers to separate distinct phases in oxygen uptake during bacterial substrate utilization studies. Bhatla and Gaudy (46) have summarized the current thinking as to the causation of such phasic oxygen uptake. Some possible factors affecting the oxygen plateau advanced by these authors are:

1. After the exogenous substrate is depleted, some cells of a predominant bacterial species lyse and release intracellular components into the system which become substrate for a multiplying secondary population. Some time may be required to develop a secondary population to an extent such that its effect is reflected in the oxygen uptake curve. An identical effect could be produced if the secondary population consists of protozoa. The oxygen uptake curve would then reflect the effects of predation.

2. Another possibility is that no secondary population is involved, that is, no change in predominant bacterial species. To justify the oxygen plateau it is necessary to postulate that some time is required for the remaining bacteria to become acclimated to the released intracellular substances.

3. Adaptation time for the utilization of stored intracellular components could also be involved.

4. As the bacteria metabolize glucose, intermediates are released into the bulk solution to which the bacteria may have to become acclimated before such substances can be utilized.

The above four hypotheses were intended for consideration where only one original exogenous carbon source is provided. It was recognized that sequential utilization of heterogeneous substrates could also cause phasic oxygen uptake.

From the preceding discussion regarding oxygen uptake during bacterial growth using glucose as a substrate, it is obvious that differences of opinion as well as differing experimental data exist in the literature. It is rather difficult to assess the data available due to the differing experimental techniques, different organisms used, and differences inherent in the measurement of oxygen uptake. In essence, possible physical, chemical, or biochemical parameters responsible for the varied values reported have not as yet been identified if, indeed, they exist.

Dispersion. Nejedly and Pelz (45) attempted to explain the differences in the first order deoxygenation constant, k_1, obtained in

bottle BOD tests and from free flowing streams by the dispersion which takes place in streams. An attempt was made to simulate the particle distribution due to longitudinal dispersion in a series of BOD tests by combining artificial sewages which had been undergoing biological degradation for different lengths of time. They concluded that the much higher k_1 rates observed in the artificially "dispersed" samples were analogous to the higher deoxygenation rates often observed in nature. However, it seems quite probable that these investigators were only observing the effects of increasing the organism concentration in the system by making up composite samples of different individual fermentations. Interestingly enough, Dobbins (47) has shown mathematically that in most instances dispersion is not a significant factor in the course of biochemical reactions and the resulting oxygen sag curves.

Turbulence. The effect of turbulence on bacterial substrate utilization has been reported qualitatively in the literature to some extent. The major obstacle confronting most investigators has been that of separation of the many variables which have the potential to affect biological systems. Unless the major variables affecting biological systems can be appropriately separated either physically or mathematically, very little of a quantitative nature can be demonstrated. The major obstacle apparently has been the lack of a tractable kinetic model.

The investigation of Isaacs and Gaudy (24) mentioned previously was designed to compare substrate utilization in turbulent and quiescent systems. The turbulence level of the simulated stream was rated on the basis of the reoxygenation coefficient. The results of the investigation

were inconclusive as far as determining the effect of turbulence on the system due to an inability to reproduce or otherwise account for differences in initial numbers of bacteria in the system. The range of turbulence considered was quite narrow and probably was not sufficient to reveal the effects of turbulence even if the differences in initial organism concentration had been overcome.

Sanders (48) reported that the velocity of the supernatant substrate across slime surfaces is an important growth factor. High velocities were found to produce a dense, tough attached slime mass, whereas lower velocities caused the population density to decrease. The effect reported in this case is a physical change rather than a biochemical change.

Kehrberger et al. (49), in an investigation primarily concerned with temperature influences on BOD progression in soluble substrates, demonstrated that the increase in substrate utilization rates when the temperature was increased was greater in a mixed system than in a quiescent system. The conclusion reached was that diffusion of substrate to the bacterial cell was the limiting step in quiescent systems such as a BOD bottle. By mixing the system, the rate of substrate utilization increased and exhibited an increase in rate more nearly that expected from a reaction limited system when the temperature was increased.

The effect of turbulence was alluded to by Symons (50) in a discussion of Kittrell's (5) work on stream analysis. Symons pointed out that stream conditions cannot be duplicated in a bottle. He further hypothesized that at low concentrations of both food and bacteria, the

rate of disappearance of organic matter may be affected by the random chance of contact between food and organisms. Also, once the contact is made, the bacterial oxidation should proceed at a rate dependent on the life processes of the organism. It is obvious that increased turbulence would increase the opportunity of contact between organism and substrate, thereby increasing the rate of substrate utilization.

Swilly et al. (51) concluded that the resistance to molecular diffusion within the liquid phase limits the overall rate of BOD exertion in the BOD bottle systems. Furthermore, it was stated that there was some indication that either glucose diffusion or the rate of glucose dehydrogenase activity is the rate limiting factor in the BOD test. No speculation as to the applicability of this concept to other substrates was presented.

Hartman (52) demonstrated that increasing the velocity of substrate flow through a tube filter coated with bacterial slime increased the rate of oxygen utilization. The effect was demonstrated using a kinetic expression based on the concepts advanced by Monod (1). From the limited detail presented by Hartman, it is not clear as to whether the transport of substrate or oxygen was rate limiting. In any event, the fact that turbulence produced a definite influence on the system was clear.

Monod (1), in an experiment designed to show that bacterial yield was a constant under different environmental conditions, presented data clearly revealing that turbulence increased the growth rate of *Escherichia coli* on glucose. However, one system was quiescent while the other was agitated in a Warburg device and insufficient data was

presented to determine whether or not the quiescent system was oxygen limited. The data presented indicated that the quiescent culture required more than 60 per cent more time to reach its maximum population as compared to the agitated culture.

Hernandez (31) indicated that increased agitation had no influence on bacterial substrate utilization as long as zero order substrate removal was observed. In this instance, the system was reaction limited and no amount of agitation could have produced an effect.

It is apparent that the effects of turbulence have been observed many times in past investigations, but no kinetic model has been applied to quantitatively describe its effect.

Past Stream Analyses

Unfortunately, past stream analysts have most often been concerned with fitting data using only the concept of first order kinetics. Deviations from the first order kinetic model have too often been considered the result of differing rates of reaeration (4) or inadequacies in analytical techniques. It is of interest to note that the Monod approach to bacterial growth kinetics appears to be directly applicable in instances where extreme deviations from first order kinetics have been observed since the effects of organism concentration can be evaluated.

The manner in which most stream studies have been conducted is important in the interpretation of the data obtained. It is quite probable that many past stream studies have used the general plan of attack widely used by governmental agencies. Sampling stations are

normally located just above the waste source and at half-day intervals for two days' time of travel below the waste source; with additional stations located at one-day intervals for an additional three to four days' time of travel.

With the above plan of attack in mind, the observations of Kittrell and Kocktizky (5) are especially pertinent. These investigators found that the low point in the oxygen sag curve was reached six hours below the waste discharge and was well into the "recovery" stage one day below the waste discharge. It was also noted that a plot of the die-off of coliform organisms, after a peak concentration had been reached, closely resembled a similar plot of the first stage BOD. Discrepancies between the oxygen sag curve and the apparent BOD removed in the stream resulted when analysis of the data was attempted. It appears that such discrepancies were inevitable since the BOD samples taken from the stream were reported to be eight-hour composites. The oxygen sag curve observed by these investigators strongly suggests that the organic waste was being removed at a much more rapid rate than can be explained by traditional concepts.

In a later work, Kittrell and Furfari (53) observed that an increase in numbers of coliform bacteria occurs after discharge of raw sewage to a stream. They also cited the work of others (54) indicating that total plate counts produced similar results. The implication of these observations is obvious. Bacterial growth takes place at the expense of a substrate which implies the utilization of oxygen. It is therefore not surprising that serious deviations from the first order kinetic model have been observed when samples have been obtained near

the waste sources. The deviations noted have been most serious when raw sewage or sewage receiving only primary treatment have been involved.

More recently, Velz and Gannon (6) observed a very rapid disappearance of BOD from the James River which was interpreted as "biological extraction" with subsequent utilization of the extracted BOD at another time. Although the theory advanced by these investigators appears to explain the data observed, there is some question as to the interpretation of the data upon which the theory is based. Normal time series BOD data represents the total amount of oxygen consumed by a bacterial population in utilizing a substrate for growth and the subsequent utilization of oxygen accompanying the autodestruction of the population. If a sample is held any appreciable length of time before the BOD test is begun, it is highly probable that significant oxygen utilization, corresponding to bacterial growth, will take place. Although Velz and Gannon (6) did not provide sufficient information to evaluate this point, the oxygen sag curve they presented suggests the possibility. The observed oxygen profile is of the type predictable from the Monod bacterial growth kinetic expressions.

Schroepter et al. (7) in an investigation of the Mississippi River near Minneapolis-Saint Paul, observed that of 250,000 pounds per day of 5-day BOD discharged, 125,000 pounds per day were satisfied within one-half day time of flow. Traditional values of the deoxygenation coefficient indicated that only 30,000 pounds per day should have been satisfied. This observation prompted them to evaluate the applicability of the classical first order kinetic model. The results of

the evaluation indicated serious discrepancies when first order kinetics were applied to four-hour time series BOD data on raw, primary and chemically treated effluents. As a result, first order kinetics and the Streeter-Phelps oxygen sag equation were abandoned in favor of an empirical equation for BOD exertion and a numerical integration technique for performing the oxygen balance. The oxygen uptake curves obtained by these investigators are identical in general shape to those exhibited later in this presentation.

It is apparent that unexplained deviations from the traditional first order approach to stream analysis exist. Based on the work of the investigators cited, it is also apparent that the method of attack and analytical techniques employed can lead to quite different conclusions.

Summary

The literature cited in this review generally supports the concepts advanced by Monod for the analysis of bacterial growth kinetics (1,9,11,14,15,16,17). Data analysis has recently been simplified to enable the constants of the Monod equations to be determined from batch data (10).

Several investigators have used systems to study bacterial substrate utilization which were open to the atmosphere (9,23,24). The results obtained by all these investigators produced responses predictable using the Monod equations. The unavailability of the Monod equations to these researchers often resulted in the lack of attainment of the objective of the study.

Of all the possible variables affecting the study of bacterial substrate utilization, it appears that the most serious is the measurement of bacterial activity. The use of enzymatic measurements appears to hold considerable promise to enable researchers to identify the effects of viable organisms (28,29,30,31).

The most serious objection to the concept that bacterial yield is not a constant for a bacterial population is the difficulty in performing accurate dry weight measurements. Present techniques are not sufficiently sophisticated to draw such a conclusion based on available data (15,25,31,34,35,36,37). Until conclusively shown otherwise, the yield factor can be used as a constant for batch systems.

Although few researchers have reported maximum bacterial growth rate constants, k^m, it appears that the k^m values obtained under a variety of environmental conditions supports the hypothesis that k^m is a true constant dependent only on the influence of temperature (1,15, 34,39,40).

The amount of oxygen utilized per unit of substrate removed during bacterial substrate utilization also appears to be a constant (25, 42,43). The differences in reported values may be normal differences produced due to differing experimental systems and measurement techniques.

Based on the information available, the effect of dispersion in most natural streams on bacterial substrate utilization can be neglected (47).

The effect of turbulence on bacterial substrate utilization has been shown qualitatively by several investigators (1,49,51,52). The

lack of a tractable kinetic model and the inability to separate the effects of other variables from that of turbulence has limited the usefulness of past investigations.

The analysis of polluted streams in the past has also suffered from the lack of an applicable kinetic model. Several past stream analyses reported in the literature strongly support the applicability of the Monod equations to the analysis of streams (5,6,7,53).

This review is seen to substantiate the use of the Monod equation in the analysis of bacterial substrate utilization in BOD bottle systems, open batch cultures, continuous flow systems, and free flowing streams.

CHAPTER III

EXPERIMENTAL APPARATUS

Batch Reactor

A batch reactor of clear Plexiglas was constructed to provide an environment to study the interactions of the biosphere, hydrosphere, and atmosphere. The dimensions and details of the reactor are shown in Figure 1. All components of the reactor were made of clear Plexiglas with the exception of the impeller shaft. The shaft was a quarter-inch diameter stainless steel rod. Two impellers were used in this investigation. One was fabricated from a piece of clear Plexiglas in the shape of a flat blade, while the other consisted of a small tin-plated fan blade. The Plexiglas blade was used to provide $k_L a$ values of 4.0 day^{-1} and below, and the fan blade was used for higher values. Two baffles as shown in Figure 1 were installed to prevent extreme surface distortion and to minimize to formation of a vortex at high mixing speeds. The galvanic cell oxygen analyzer (D.O. probe) was mounted in the same position throughout this investigation.

Temperature Control

Two arrangements for temperature control were used during the conduct of this investigation. The initial arrangement utilized a surplus, portable, photographic water bath unit which consisted of a water bath, recirculating water pump, heat exchanger, and a freon refrigeration unit with thermostatic control.

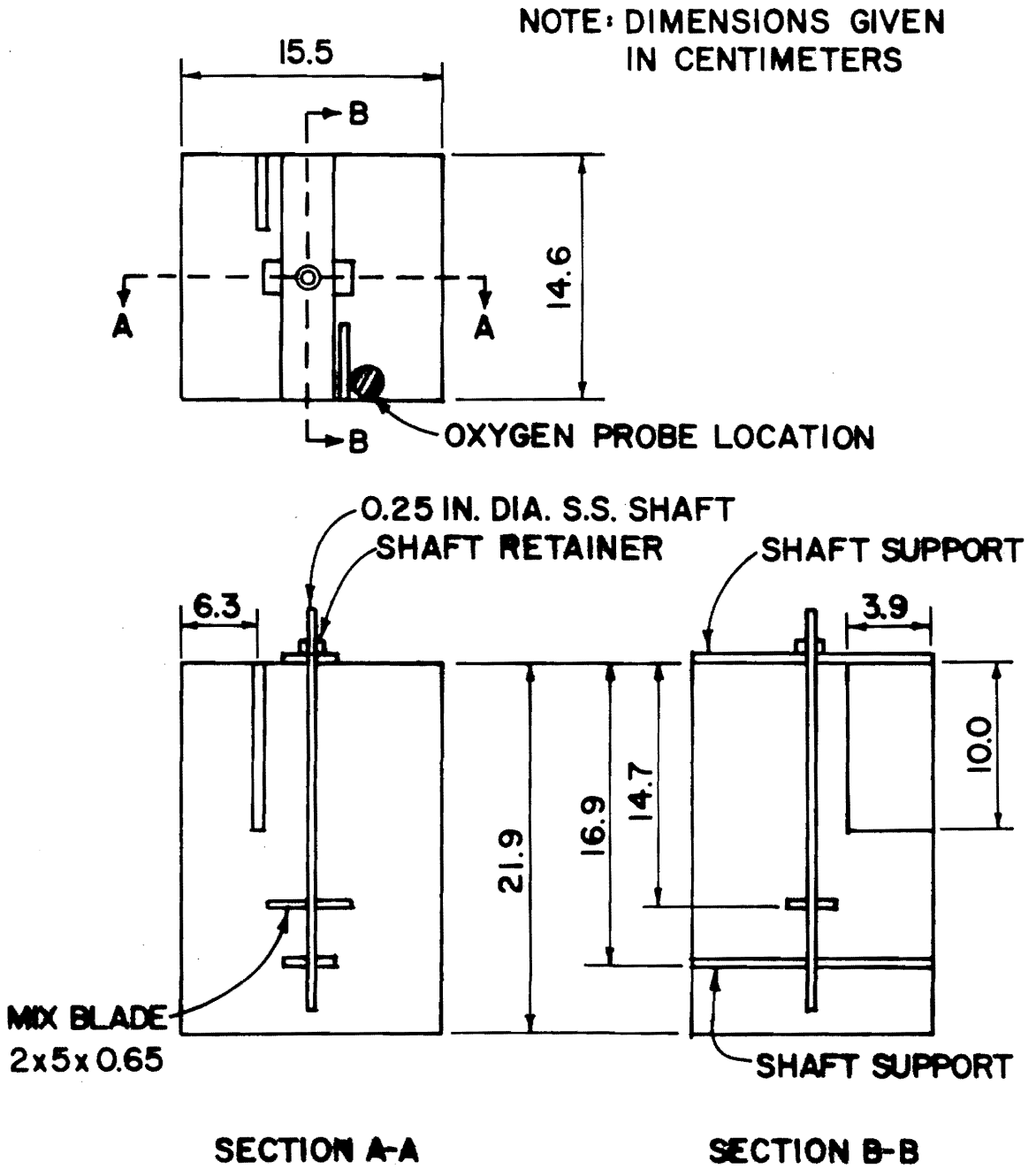


Figure 1. Details of Experimental Reactor

It was found that the temperature in the water bath varied from 19°C to 21°C, while the temperature in the reactor was quite constant. The contents of the reactor varied to no more than ±0.2°C during the course of any particular experiment.

The second arrangement consisted of a constant temperature water bath obtained from American Instrument Company (Model 4-8600) and was capable of holding the reactor contents at a temperature such that little or no variation in temperature was noted during the conduct of any particular run. This piece of equipment accomplished temperature control through the use of both heating and cooling systems which were regulated thermostatically to provide the desired temperature.

Mix Speed Control

The propeller shaft in the reactor was attached to a variable speed transmission (Zero Max Company) which was driven by a 1/6 hp electric motor. This arrangement was capable of providing any desired speed of mixing between zero and 400 rpm and proved to be both flexible and reliable.

Galvanic Cell Oxygen Analyzer

A commercially available lead-silver galvanic cell oxygen analyzer (D.O. probe) was used to monitor and record the changes in oxygen levels throughout this investigation. The use of equipment of this type is well documented as to its applicability and usefulness (55,56). With proper procedure and technique, this equipment proved to be most satisfactory.

Recording Devices

Two recorders were used during this investigation. The first was a Dynatronic Instruments Company Graphi-corder-10. The minimum chart speed available on this instrument was 16 inches per hour and was abandoned when a recorder with slower chart speeds was obtained. The second recorder used was a Fisher Scientific Integrating Laboratory Recorder. Chart speeds of 0.5 to 8 inches per minute or inches per hour were available with this instrument and it was used for the bulk of the experiments performed. Since both recorders used recorded voltage, it was necessary to modify the input to both recorders from the D.O. probe through the use of series variable resistors wired in parallel with the input to the recorder. By manipulation of the resistors and the zero adjustment of the recorders, the recorded output of the D.O. probe could be made to very nearly correspond to the oxygen concentration of the system used.

CHAPTER IV

ANALYTICAL METHODS

Dehydrogenase Activity--Test Development

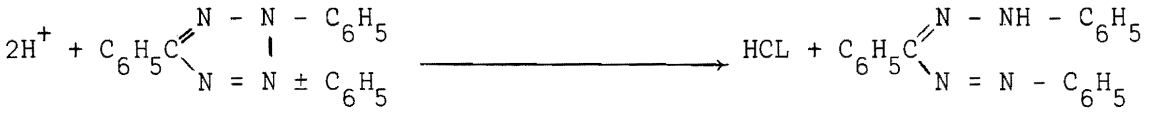
The bacterial systems used in this research were of necessity quite dilute, that is, 1-25 mg/l dry weight of viable organisms. This, coupled with the desirability of measuring only viable organisms, led to consideration of utilizing a test which might, in fact, measure the concentration of only viable organisms. In consideration of the enzymatic nature of all biochemical reactions, it was decided to employ a measure of enzymatic activity. Lenhard et al. (29) reported a method of measuring the dehydrogenase activity of activated sludge systems. They reported that the work of others indicated a close correspondence between the dehydrogenase activity of sludges and the counts of viable bacteria in them. However, since activated sludges contain quite large populations of viable bacteria, it was necessary to modify the test as presented by Lenhard et al. (29) considerably to adapt it to the systems studied here.

Basically, the test consists of the reduction of triphenyltetrazolium chloride (TTC) by a dehydrogenase to triphenylformazan (TF) which is red in color and can be measured spectrophotometrically. The color produced is proportional to the dehydrogenase activity of the sample.

RH_2 DEHYDROGENASE $R + 2H^+$

SUBSTRATE ⟶ OXIDIZED SUBSTRATE

(e.g. $CH_3CH(OH)COOH$) (e.g. $CH_3COCOOH$)



TTC
(Colorless)

TF
(Red Color)

From the reaction given above, it can be seen that to develop the color, the TTC must act as the hydrogen acceptor or no color will be developed.

It was found that in samples of low bacterial activity, no (or little) color was developed. From this observation it was concluded that oxygen present in the sample was acting as the hydrogen acceptor rather than the TTC. Subsequently, the addition of sodium sulfite and bubbling nitrogen through the sample were evaluated.

A mixed bacterial culture was obtained by innoculating a 1000 mg/l glucose solution fortified with inorganic nutrients (see Appendix 2) from a culture which was maintained on nutrient agar slants at 30°C. The culture was then allowed to develop at 20°C while being mixed on a magnetic stirring device. At the end of approximately 48 hours, the culture was harvested by centrifuging in an International Clinical Centrifuge (Model CL) for 3-5 minutes followed by one wash with a 0.9 per cent phosphate buffered saline solution. Details concerning the wash solution are presented in Appendix 2. After washing, the solids remaining were resuspended in distilled water. Dilutions of the washed

sample of 100, 75, 50, 25, 12.5 and 6.5 per cent of the sample were prepared by diluting to the appropriate amount with distilled water and determining the dehydrogenase activity of each using the standard reagents and procedure as detailed later, but without the addition of sulfite or nitrogen gas. In addition to these determinations, the dry weight of the 100 per cent sample was determined by inactivating the culture by the addition of one milliliter of formaldehyde to each of four samples which were subsequently evaporated over a water bath, dried at 103°C for three minutes, and cooled in a desiccator. As shown in Figure 2, the average dry weight concentrations of these samples of 204.5 mg/l corresponded to a dehydrogenase activity of optical density 2.0. The non-linearity at low values was taken to indicate that oxygen was present and competed with the TTC as a hydrogen acceptor which subsequently prevented the TF from being formed. This possibility was investigated further.

Since it was anticipated that reliable results would be needed from the dehydrogenase test at optical densities below 0.20, the addition of sulfite to the sample during the test was investigated. As shown in Figure 3, results from another sample treated in a similar manner as described earlier indicated that the sulfite itself caused a small amount of color to develop which predominated below the 0.1 O.D. value. The use of sulfite in the test procedure was subsequently abandoned.

An apparatus to enable continuous bubbling of nitrogen through the samples was constructed using a manifold and laboratory tubing.

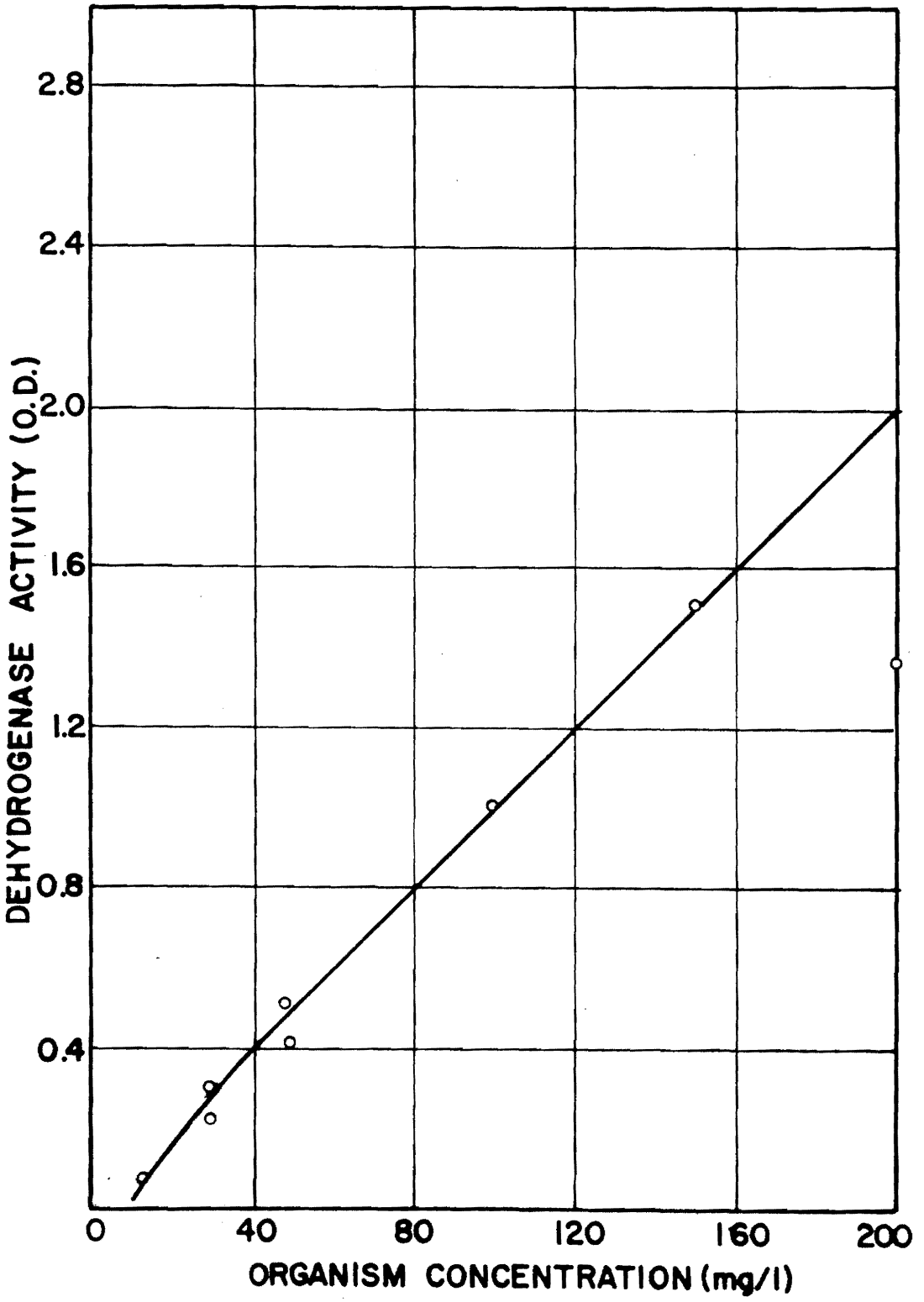


Figure 2. Dehydrogenase Activity as a Function of Organism Concentration

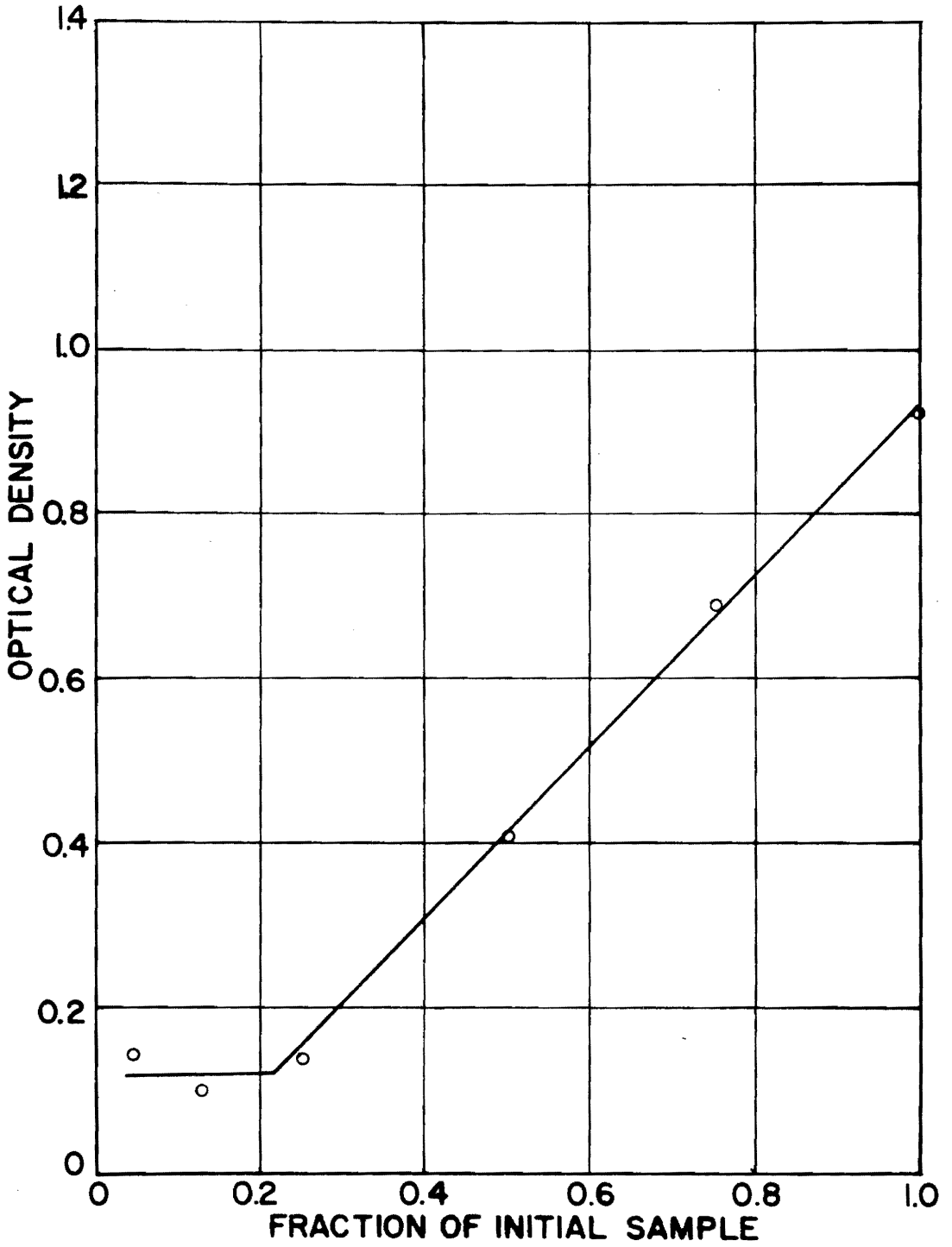


Figure 3. Optical Density as a Function of Organism Concentration for Dehydrogenase Test Using Sulfite

Using this equipment and a low density culture, the results shown in Figure 4 were obtained. These results indicated that a linear relationship between bacterial density and dehydrogenase activity essentially to zero was obtained. Based on the investigations described above, the use of nitrogen to exclude oxygen from the test system and to insure proportional color development was verified.

An observation of interest not affecting the use of the test or the conduct of this investigation was the microscopic examination of samples taken from the experimental reactor during a test. It was noted that before the alcohol was added to the solution to put the color developed into solution, the color appeared to be located on or in the biomass in the test tubes. Upon subsequent examination under the oil immersion lense of a microscope, it appeared that the color was entirely located on or in the individual bacterial cells. Several distinct types of bacterial forms were observed. Most often, the predominant types seen were rods of various sizes. As a rule, the rods were either completely red or contained no color at all. The cells containing no color could be seen either in a mass or by using a negative staining technique. The cells containing no color were interpreted as containing no dehydrogenase enzyme and were probably not viable. Coccus cells observed contained red granules which appeared to be contained within the cell.

Martin (57) in a paper on bacterial protoplasts, listed succinic, formic, glycerophosphate, and ketoglutaric dehydrogenase as being located in the cytoplasmic membrane of bacteria. Isocitric dehydrogenase was said to be located in the soluble cytoplasm while malic and

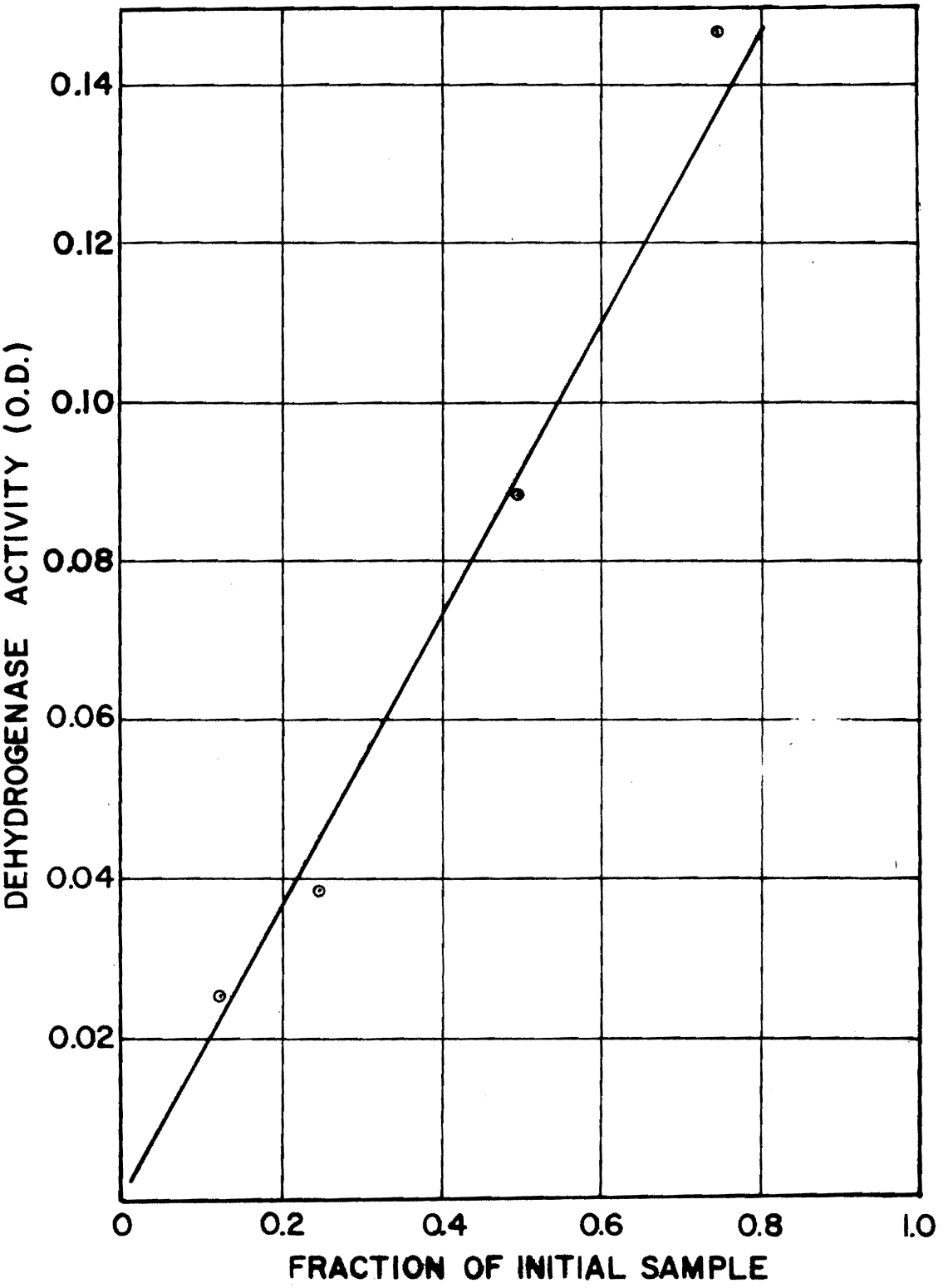


Figure 4. Dehydrogenase Activity of a Dilute Culture as a Function of Organism Concentration

lactic dehydrogenase can appear in both locations.

Malatyan and Biryuzoza (58) were able to locate the sites of dehydrogenase activity of bacterial cells by using TTC to form formazan on the surfaces upon which dehydrogenases were located. The cell was then thin sliced and viewed under an electron microscope to show that there was selective deposition of formazan on the dehydrogenase surfaces.

In subsequent experience with this test using sewage several days old, it was noted, microscopically, that some color appeared to be in solution. A possible cause of this occurrence is release of intracellular enzymes into the solution upon cell lysis. If this is true, then it may not be possible to obtain a valid relation between the dry weight of "viable" organisms and the results of the dehydrogenase test presented here in "old" cultures. The extent of effect upon the general applicability of the test to bacterial systems is not known. However, Tengerdy et al. (59) recently found that the color produced by cultures of *E. coli* and *Staphylococcus aureus*, in a nutrient solution containing TTC, was directly proportional to the viable populations as determined by plate counts.

Detailed Procedure--Dehydrogenase Test

1. Add eight milliliters of the sample to one milliliter of 0.05M tris (hydroxymethyl) aminomethane hydrochloride (tris-HCL).

2. Bubble nitrogen through the solution to remove any oxygen present and continue throughout the remainder of the test.

3. Incubate the sample at 37°C for five to ten minutes to insure uniform temperature.

4. Add one milliliter of the TTC-glucose reagent to the solution and continue the incubation for an additional 60 minutes. The test should be protected from strong light at all times.

5. At the end of the incubation time, the color development is stopped by the addition of one milliliter of formaldehyde.

6. The sample is then diluted with 95 per cent ethanol to either 35 or 50 milliliters, depending upon the extent of color development. A graduated test tube is quite useful for this step.

7. Allow the sample to stand in darkness for 30 minutes to insure complete solution of the color.

8. If an inordinate amount of particulate matter is in the sample, filtration may be necessary. Either paper or glass-fiber filters are adequate.

9. The optical density of the sample should be measured at 483 mμ against a blank to which one milliliter of distilled water was added rather than the TTC-glucose reagent. A pair of matched cells with a ten centimeter light path is useful for this test.

Reagents

1. Tris-HCL Buffer 0.05M. Dissolve 6.037 grams of tris (hydroxymethyl) aminomethane and add 20 milliliters of 1N HCL in one liter of distilled water.

2. TTC-Glucose Reagent (30). Dissolve 0.20 g. of triphenyltetrazolium chloride (TTC) and 1.50 g. of glucose in 100 ml of distilled water. This reagent should be stored at 2°C and prepared weekly.

3. Triphenylformozan Standard. Dissolve 0.300 g. of triphenylformozan (M.W. 300.4) in 500 ml of ethanol and allow it to stand in

darkness overnight. Suitable dilutions of this standard can be used to obtain a standard curve of optical density versus μmoles of TF per ml.

4. Use either 95 per cent or absolute ethanol.

Glucose Determinations

Procedure Using Worthington Biochemical Corporation, Freehold, N. J., Reagents

1. Prepare a suitable concentration working standard from a 1.0000 gm/l standard glucose solution.

2. Add four milliliters of the glucostat reagent to each of three test tubes.

3. Add 4.0 ml of distilled water to the first tube, 4.0 ml of the working standard glucose solution to the second tube, and 4.0 ml of unknown to the third tube.

4. Incubate the three tubes at 37°C for 30 minutes.

5. After incubation add one drop of 4M HCL to each tube to stop the color reaction.

6. Measure the per cent transmittance of the standard solution and unknown sample against the blank at 400 mμ in a spectrophotometer.

7. The concentration of the unknown may be computed as:

$$\left[\frac{\log 100 - \log \%\ T \text{ unknown}}{\log 100 - \log \%\ T \text{ standard}}\right] \times \text{Concentration Standard} = \text{Concentration of the Unknown}$$

Reagents

1. Glucostat Reagent:

   a. Dissolve the powdered chromogen by adding distilled

water to the container vial.

b. Dissolve the powdered glucostat by adding distilled water to the container vial.

c. Add both the dissolved chromogen and glucostat to a 100 ml graduate. Rinse each vial with distilled water and add the wash water to the graduate.

d. Dilute the solution to 50 ml. The solution may become colored upon standing a few hours. This can be prevented somewhat by storing in darkness in a refrigerator.

2. HCL, 4M:

a. Dilute 34.0 ml of concentrated HCL to 100 ml with distilled water.

3. Standard Glucose Solution:

a. Dissolve 2.5 gm of benzoic acid in one liter of boiling distilled water. After dissolving the benzoic acid, allow the solution to cool.

b. Dissolve 1.0000 gm of reagent Dextrose in the benzoic acid solution and dilute to one liter in a volumetric flask. This solution should be stable for at least one year at room temperature.

CHAPTER V

EXPERIMENTAL PROCEDURE

Operation

Reaeration Rates

To insure linearity between the D.O. probe output and the oxygen level of the system under measurement, the span of the recorder was set by use of the zero adjustment of the recorder and the resistor system wired to the D.O. probe. A saturated solution of sodium sulfite was used to establish the zero point for the oxygen to which the recorder was adjusted. After adjusting the recorder, the D.O. probe was carefully washed to remove all traces of the sulfite and then installed in the reactor. The probe was clamped against the same baffle in all tests with the tip of the probe being identically positioned each time.

Four liters of distilled water stored at 20°C for several days in an open glass container was added to the reactor during each test. Gaseous nitrogen was then bubbled into the reactor contents through a diffuser to remove the dissolved oxygen from the system. When the recorder indicated that the oxygen level was quite low, the flow of nitrogen was discontinued and the diffuser removed from the reactor. The reactor was then mixed at the desired speed and allowed to reaerate. From the resulting continuous recording of output from the D.O. probe, dissolved oxygen concentration in the reactor at any time could be calculated. When the dissolved oxygen level in the reactor was

essentially constant (near saturation), two samples were taken from the reactor. The dissolved oxygen concentration of these two samples was determined by the Winkler method (60). The sensitivity coefficient for the probe under the specific conditions of each test could then be determined and from this all other values of the probe output could be converted to dissolved oxygen concentrations. By assuming values for the saturation D.O. concentration and using the linear form of the well-known equation relating the rate of reaeration to the deficit of oxygen in the system, the overall oxygen transfer coefficient ($k_L a$) was determined.

$$\frac{dC}{d\theta} = K_L a \, (C_s - C_\theta) \tag{2}$$

where $\frac{dC}{d\theta}$ = time rate of change of D.O. concentration

$K_L a$ = overall oxygen transfer coefficient

C_s = D.O. saturation value for the water in the system

C_θ = D.O. concentration at some time, θ.

Integrating:

$$\int_{C_o}^{C_\theta} \frac{dC}{C_s - C_\theta} = K_L a \int_o^\theta d\theta \tag{3}$$

$$- \ln (C_s - C_o)\Big|_{C_o}^{C_\theta} = K_L a \, \theta \tag{4}$$

Or:

$$\ln\left[\frac{C_s - C_\theta}{C_s - C_o}\right] = -K_L a\theta \qquad (5)$$

Rearranging into a linear plotting form:

$$\ln (C_s - C_\theta) = -K_L a\ \theta + \ln (C_s - C_o) \qquad (6)$$

Or:

$$\log (C_s - C_\theta) = -k_L a\ \theta + \log (C_s - C_o) \qquad (7)$$

Where:

$$k_L = K_L/2.303$$

By plotting log $(C_s - C_\theta)$ as a function of θ, and assuming values of C_s until a straight line was obtained, the slope of the resulting plot yielded the value of $k_L a$ for the particular test. The results of these determinations are presented in Figure 5. The values of C_s which were assumed varied from 8.7 to 8.9 mg/l which represents a range of from 95 per cent to 98 per cent of the generally accepted value of 9.14 mg/l at 20° C (60).

The mixing speed was determined by physically counting the number of revolutions per unit of time (usually 15 to 30 seconds) a number of

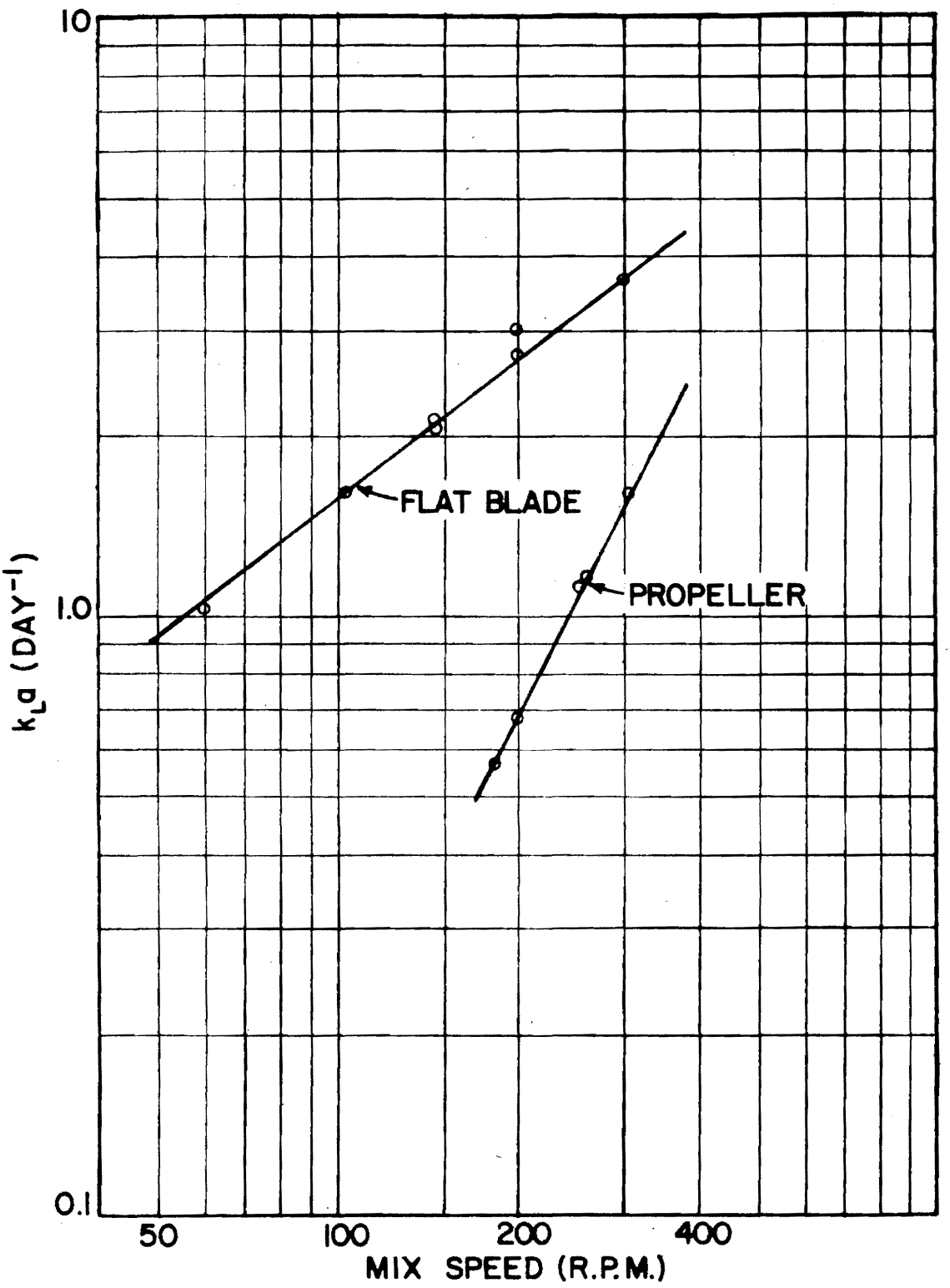


Figure 5. k_La as a Function of Reactor Mix Speed for Reactor Volume of 4.0 Liters

times, converting these figures to revolutions per minute (r.p.m) and averaging the results. With practice, some facility using this method was developed and speeds as high as 300 r.p.m. could be determined with good reproducibility.

Substrate Utilization Studies

Preparation for each test was begun several days in advance. One gram of reagent grade glucose, the inorganic nutrient stock solutions (see Appendix 2) and sufficient distilled water to bring the final volume to one liter was added to a two liter erlymeyer flask. Several loops of a bacterial culture started from activated sludge and maintained on nutrient agar incubated at 30°C was added to the solution. The flask was then placed in a 20°C incubator on a magnetic stirring device and mixed rapidly with a plastic coated mix bar. At the end of from 24 to 48 hours, the culture was harvested by centrifuging the sample for 3-5 minutes per sample, decanting the supernatant, resuspending the bacterial solids in 0.9 per cent phosphate buffered saline solution (see Appendix 2), and centrifuging again for 3-5 minutes. Following this, the supernatant was again decanted and the bacterial solids resuspended in a small amount of distilled water and added to a volume of one liter in a graduate cylinder which was being continuously mixed on a magnetic mixer. From 3 to 3.5 liters of distilled water containing the desired amount of glucose to give a final concentration at 4.0 liters volume of 50 mg/l and 20 ml each of the stock solutions of inorganic nutrients (see Appendix 2) was added to the reactor during the time when bacterial "seed" harvesting was taking place. The D.O. probe was installed in the reactor and the reactor contents were mixed at the speed desired

for the test. In this manner both the temperature and the D.O. level of the system were allowed to equilibrate somewhat before the bacteria were added to the system. When the bacterial harvesting was completed, the volume of the seed was raised to a known amount and samples were taken for dehydrogenase activity measurements. Immediately following this, the "seed" solution was diluted to within 100 ml of a volume sufficient to bring the final volume in the reactor to 4.0 liters. Distilled water ice was then added to adjust the temperature of the "seed" solution to 20°C and the final solution was added to the test reactor. Samples for glucose concentration and dehydrogenase activity determinations were taken immediately to determine the initial conditions of the test. Thereafter, the sampling program was guided by the resulting depletion of oxygen from the system. Each time a glucose determination was made, a known standard glucose solution was analyzed. It was found that different reagent solutions prepared throughout any one test gave slightly different results and that this problem could be avoided by analyzing a standard solution each time the reactor was sampled. After the depletion of the exogenous substrate and the resulting deoxygenation and reoxygenation which took place, the test was terminated by taking two samples for oxygen determination by the Winkler method in order to calibrate the D.O. probe for the run.

CHAPTER VI

EXPERIMENTAL RESULTS AND DISCUSSION

Results

The experimental results obtained are presented in Table 3, Figures 6 through 19 and Appendix 3. The plots of substrate concentration, organism concentration, D.O. concentration, and cumulative oxygen uptake as a function of time are presented to demonstrate the first objective of this research, that is, the applicability of the Monod nutrient equation to the analysis of substrate utilization by a heterogeneous bacterial population in a batch system.

Figure 19 is presented to illustrate the influence of turbulence on bacterial substrate utilization observed in this investigation. Equation (8) is the result of a least squares fit of the experimental data presented in Figure 19.

$$K = (7.84)\ 10^{-0.01115\ k_L a} \tag{8}$$

The above equation represents the quantitative dependence of the saturation constant, K, on the turbulence level, as measured by $k_L a$, observed in this investigation.

Table 3. Summary of Results

Test No.	$k_L a$ ($hr.^{-1}$)	X_o^n (mg/l)	X_o^o (mg/l)	a (mg/l^{-1})	Y^o (mg/mg)	k^m (hr^{-1})	K (mg/l)	O_2 Uptake (mg/mg)
Closed System		-	-	-	-	-	-	0.294
3	2.45	-	-	-	-	-	-	0.260
5	2.40	50.0	10.0 9.1	0.05	0.5 0.45	0.340	7.80	0.273
6	3.00	49.0	7.6 6.4	0.08	0.61 0.51	0.337	7.20	0.294
7	1.60	19.4	4.2*	0.10	0.42	0.340	7.95	0.340
8	21.4	47.0	-	0.22	-	0.350	4.65	-
9	5.65	50.0	0.98*	0.38	0.37	0.350	6.70	0.344
10	8.26	48.5	-	0.03	-	0.354	5.20	0.416
11	8.26	47.5	2.96 4.22	0.08	0.24 0.34	0.326	6.80	0.405
12	6.80	49.5	2.2*	0.20	0.45	0.328	6.30	0.304
13	11.10	50.0	-	2.00	-	0.310	5.85	0.480
14	11.67	47.0	-	0.24	-	0.330	6.20	-
15	16.00	50.0	-	0.40	-	0.337	5.20	-
*Average of 3				Average Values	.043	0.336		.342

Where:

$k_L a$ = Rate of reaeration

X_o^n = Initial substrate concentration

X_o^o = Initial organism concentration

$a = Y^o/X_o^o$

Y^o = Bacterial yield

k^m = Maximum bacterial growth rate

K = Substrate saturation constant

O_2 Uptake = Ratio of oxygen used to amount of substrate removed.

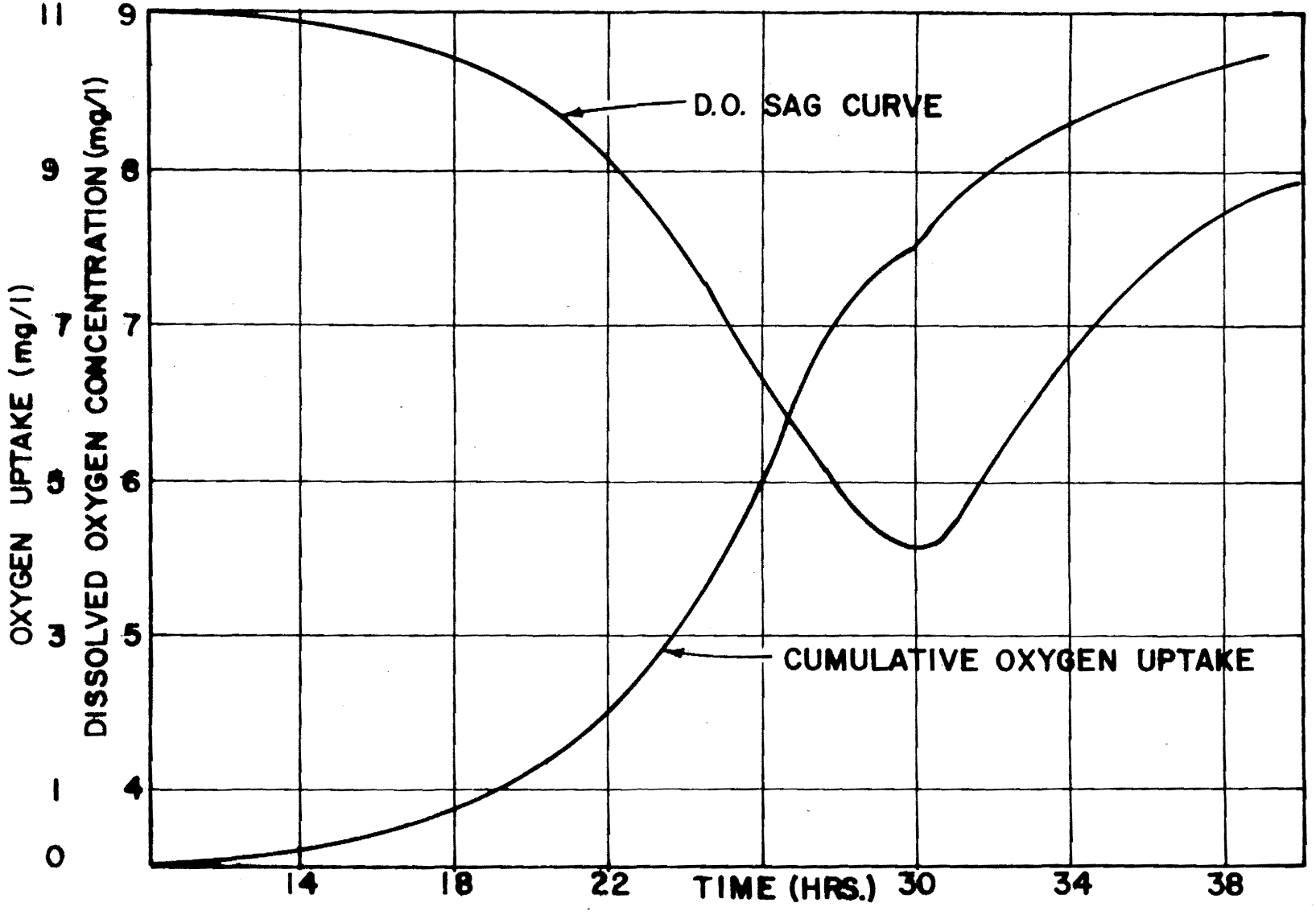


Figure 6. Dissolved Oxygen Concentration and Oxygen Uptake as a Function of Time, Test No. 1

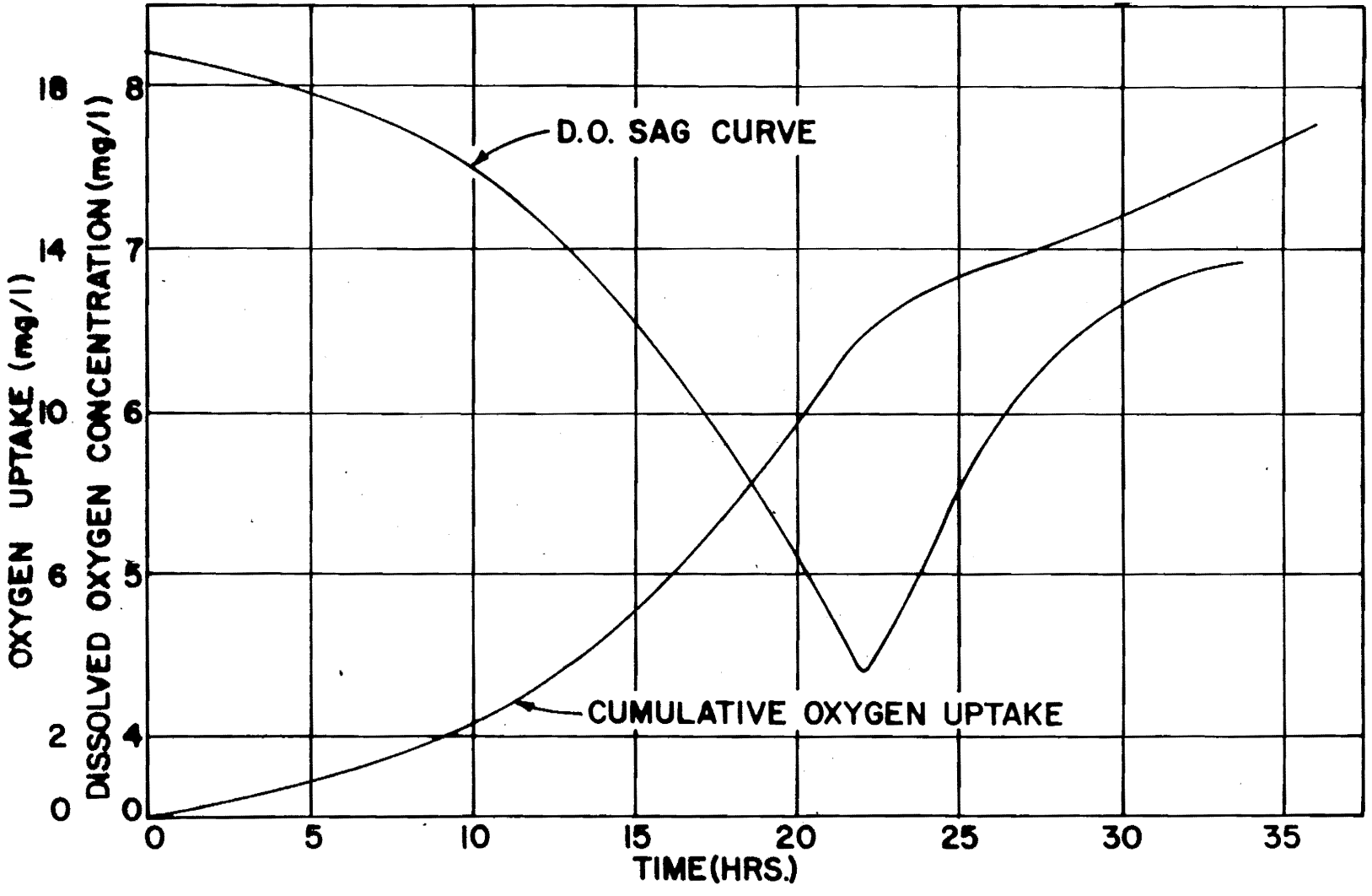


Figure 7. Dissolved Oxygen Concentration and Cumulative Oxygen Uptake as a Function of Time. Test No. 3

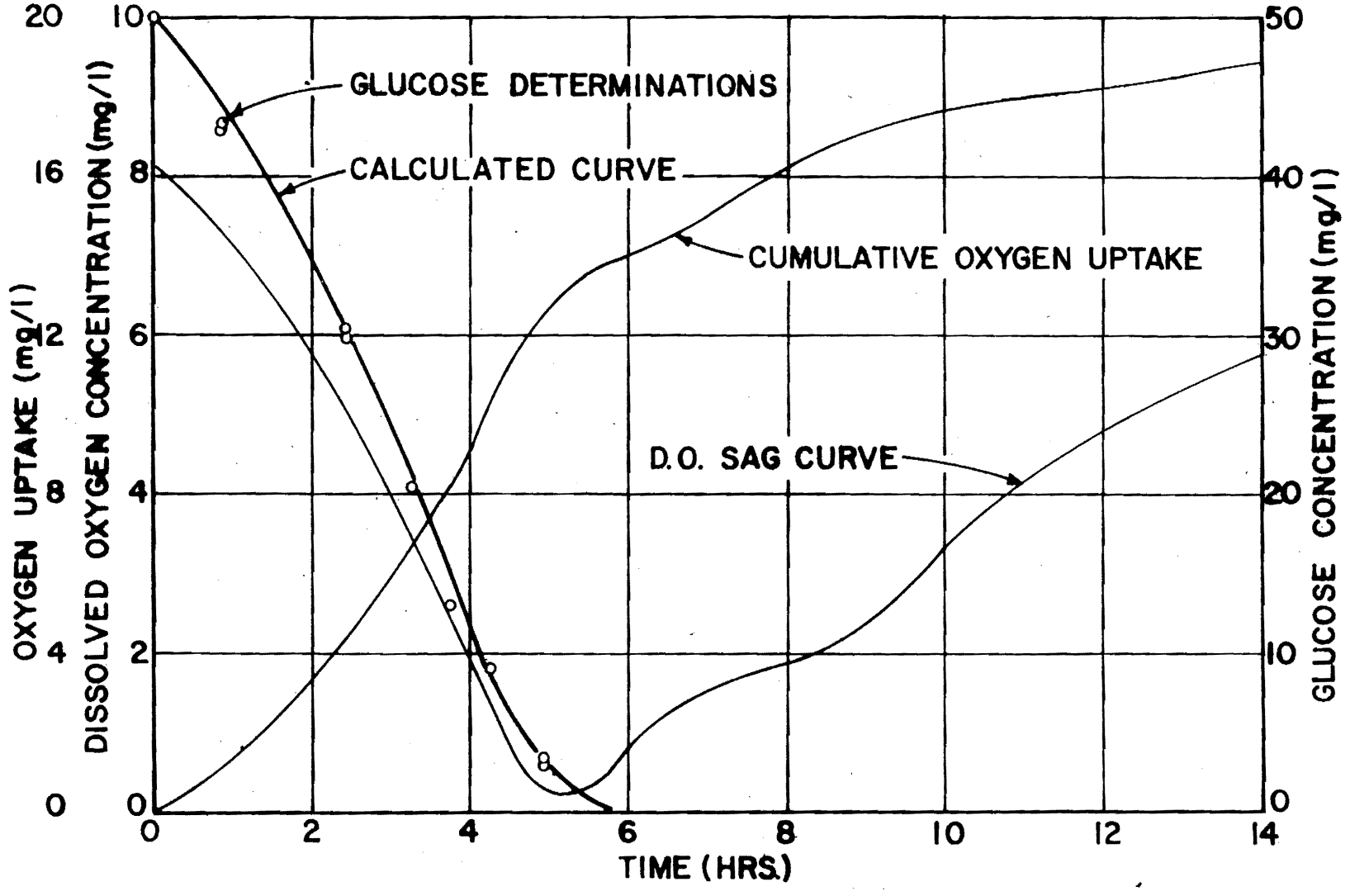


Figure 8. Glucose Concentration, Dissolved Oxygen Concentration and Oxygen Uptake as a Function of Time. Test No. 5

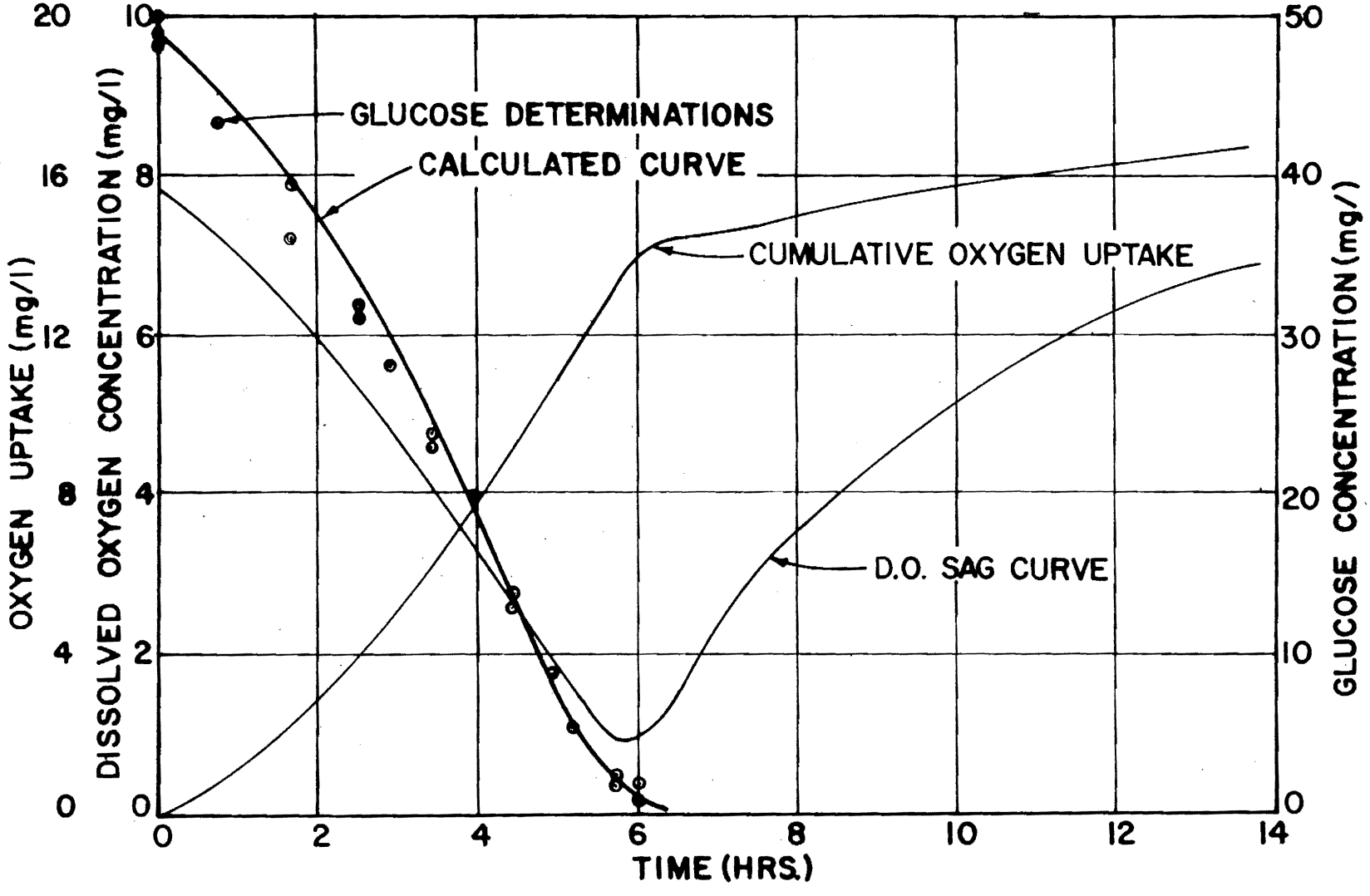


Figure 9. Glucose Concentration, Dissolved Oxygen Concentration and Oxygen Uptake as a Function of Time. Test No. 6

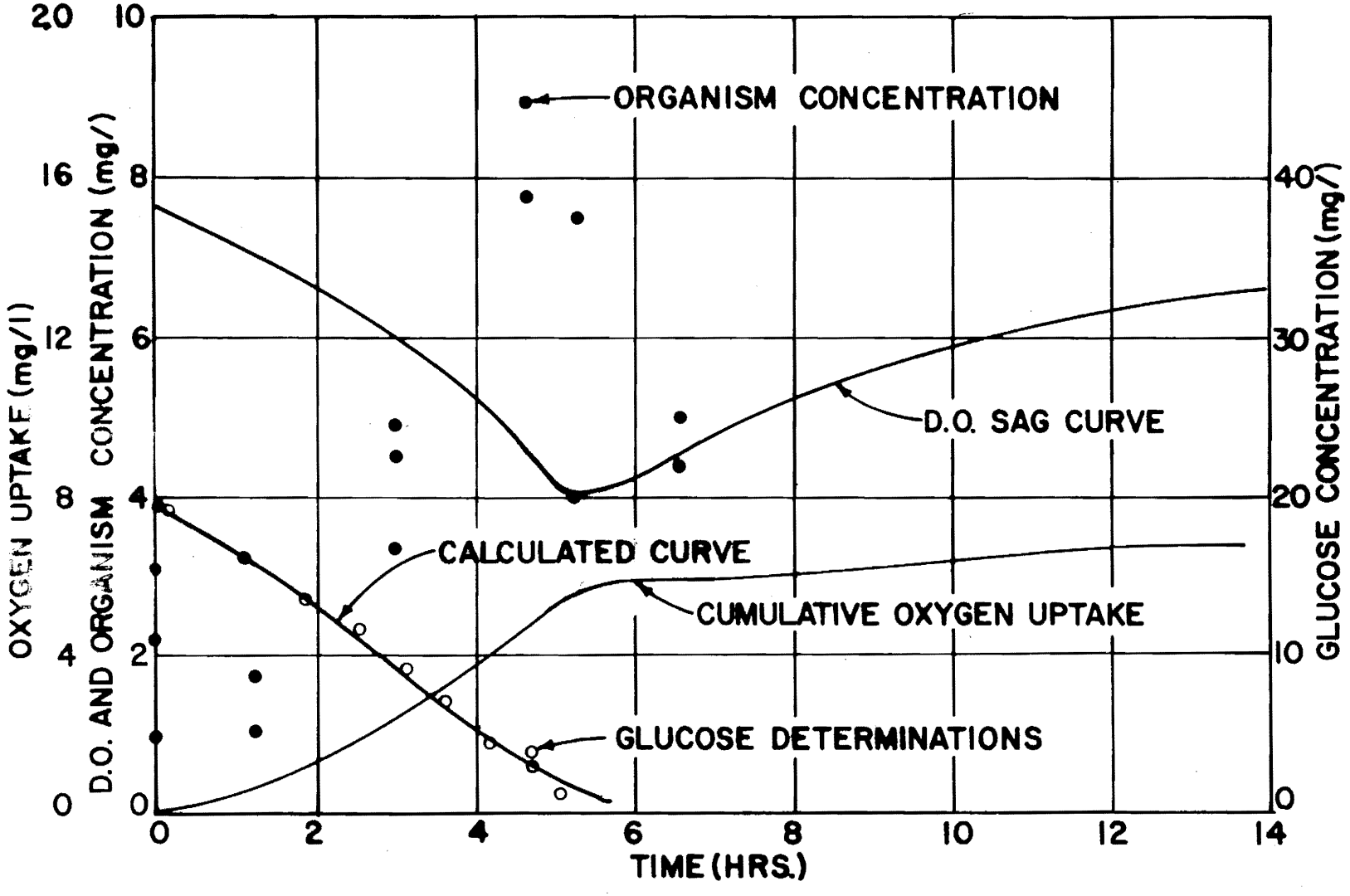


Figure 10. Oxygen Uptake, Glucose, D.O., and Organism Concentration as a Function of Time. Test No. 7

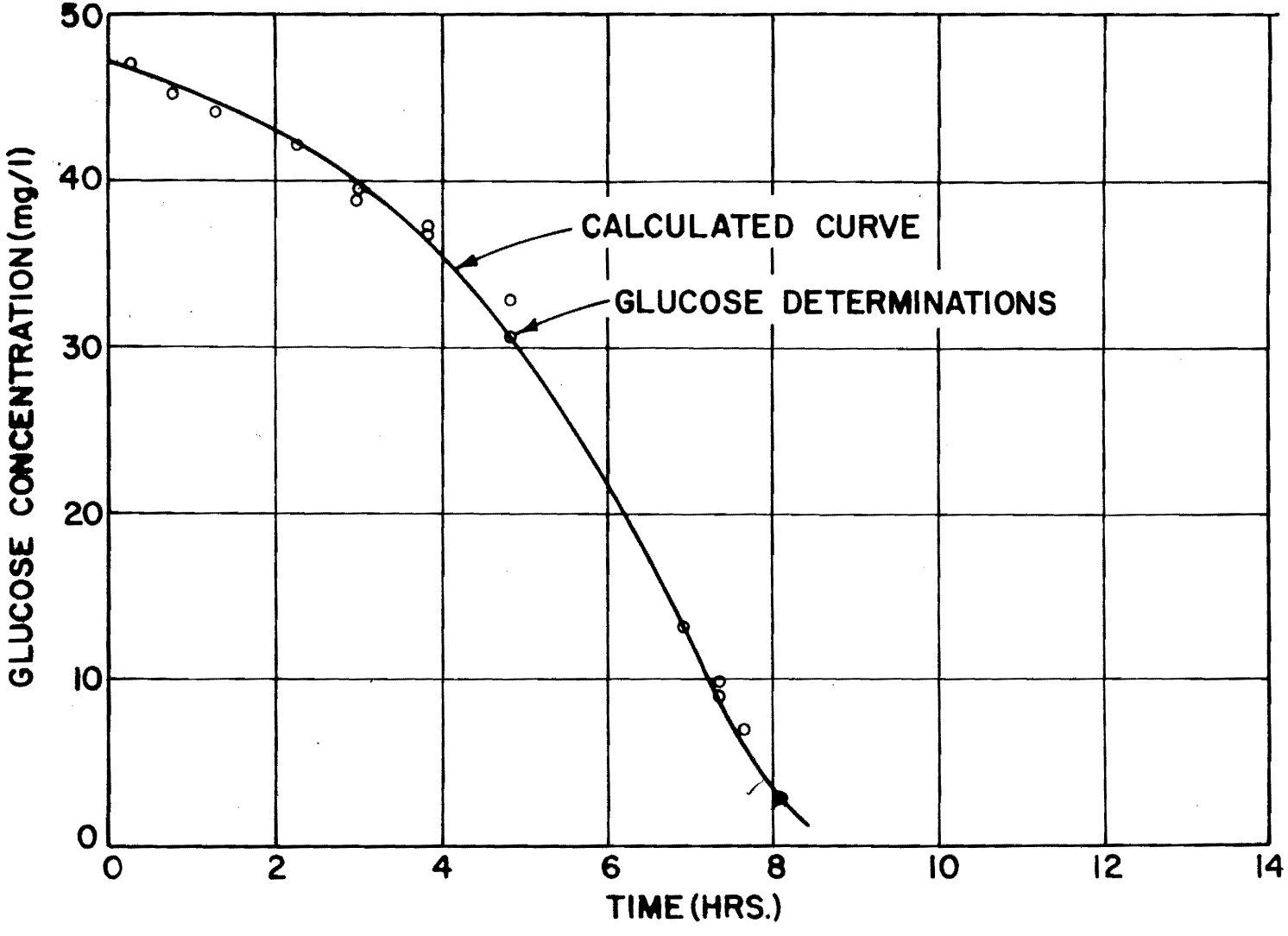


Figure 11. Glucose Concentration as a Function of Time. Test No. 8

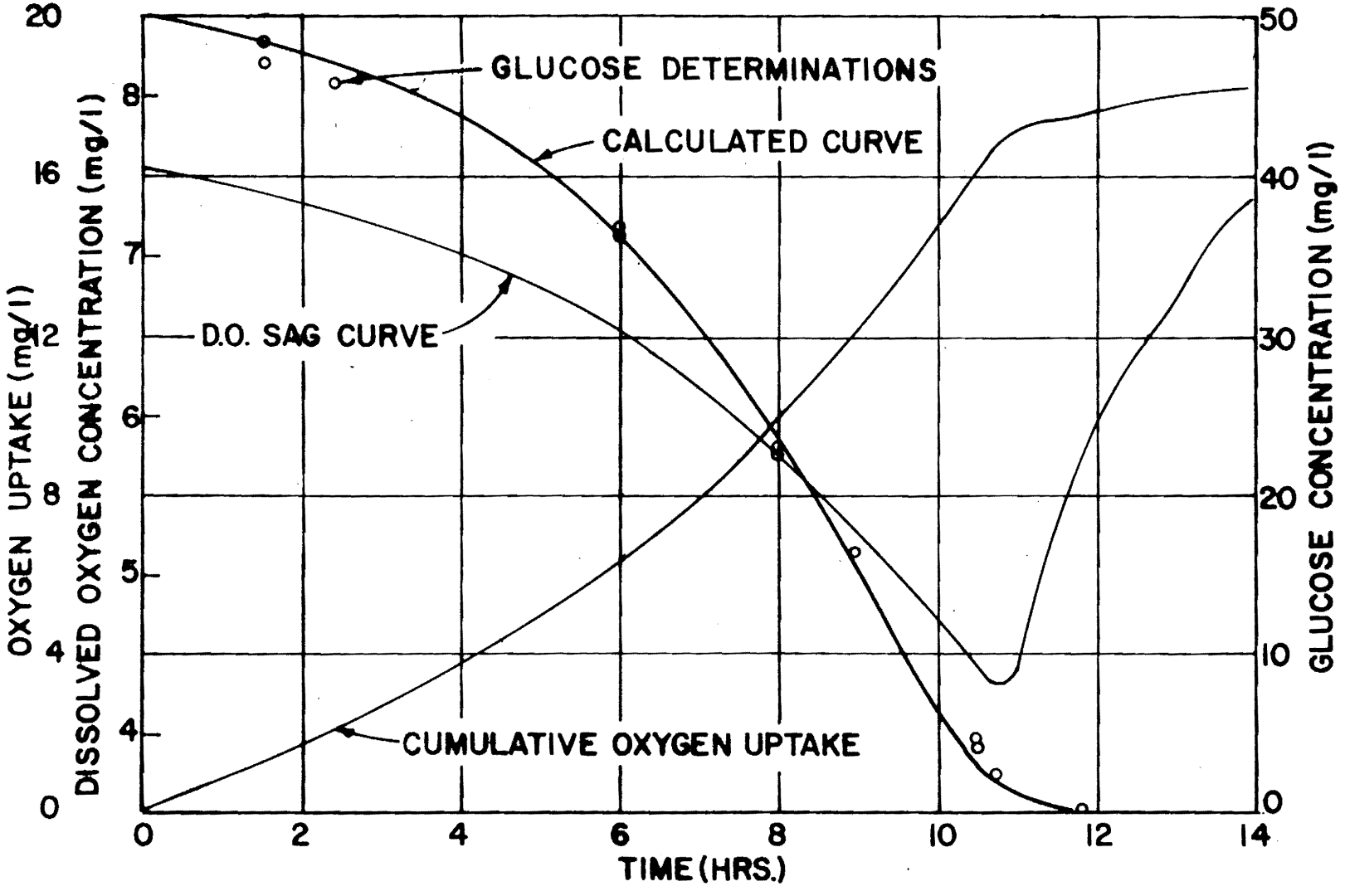


Figure 12. Glucose Concentration, Dissolved Oxygen Concentration, and Oxygen Uptake as a Function of Time. Test No. 9

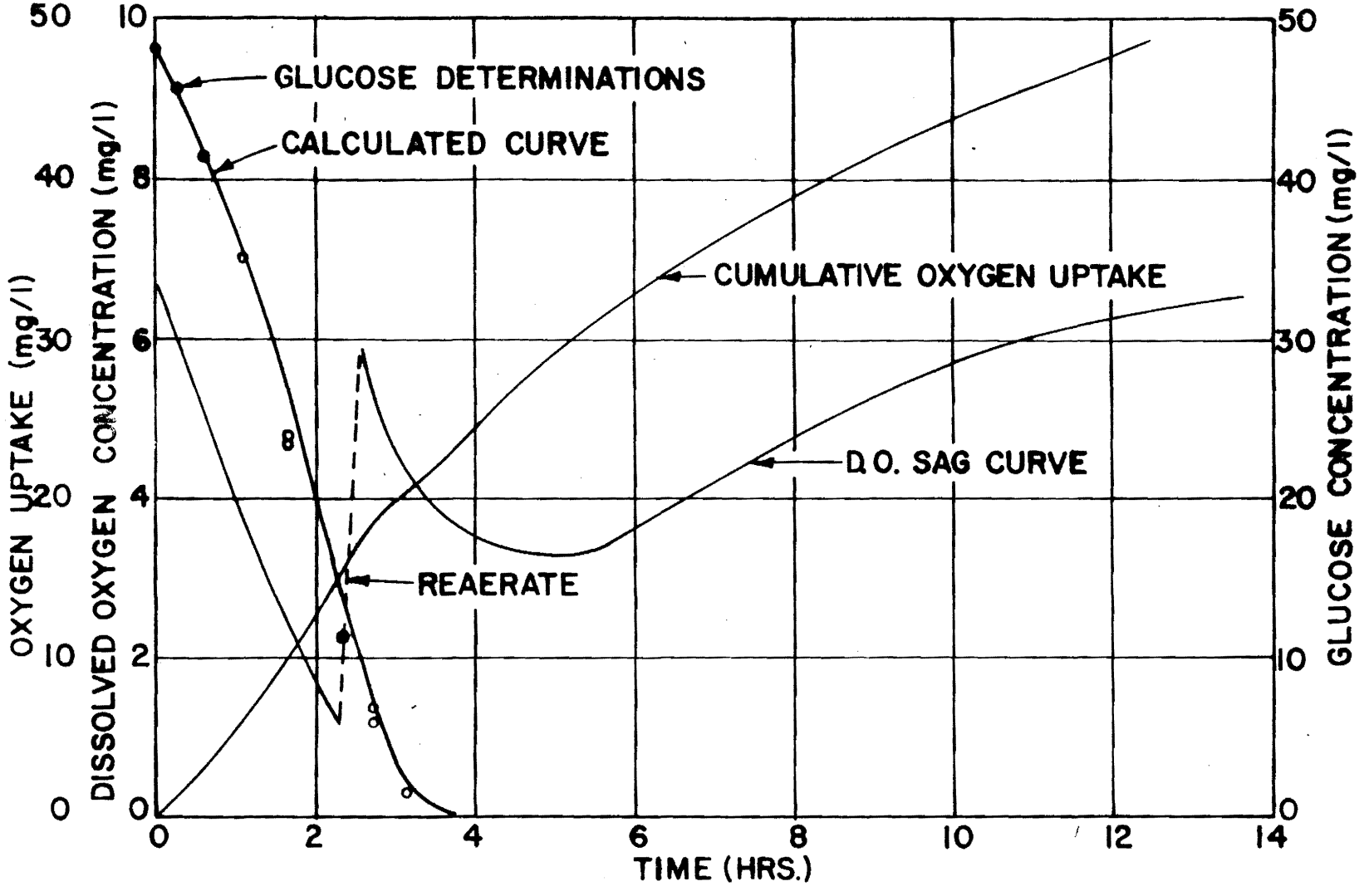


Figure 13. Glucose Concentration, Dissolved Oxygen Concentration, and Oxygen Uptake as a Function of Time. Test No. 10

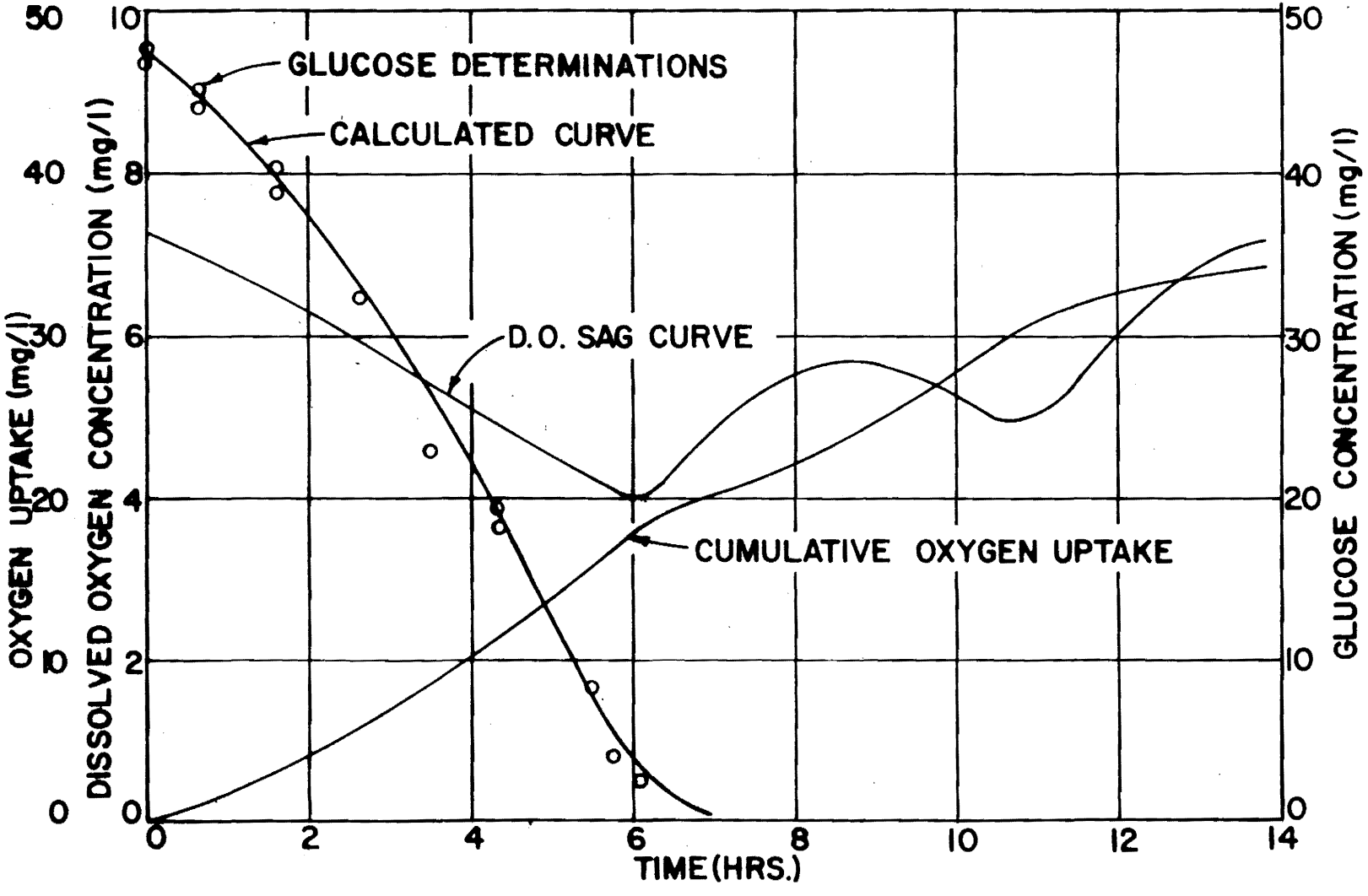


Figure 14. Glucose Concentration, Dissolved Oxygen Concentration, and Oxygen Uptake as a Function of Time. Test No. 11

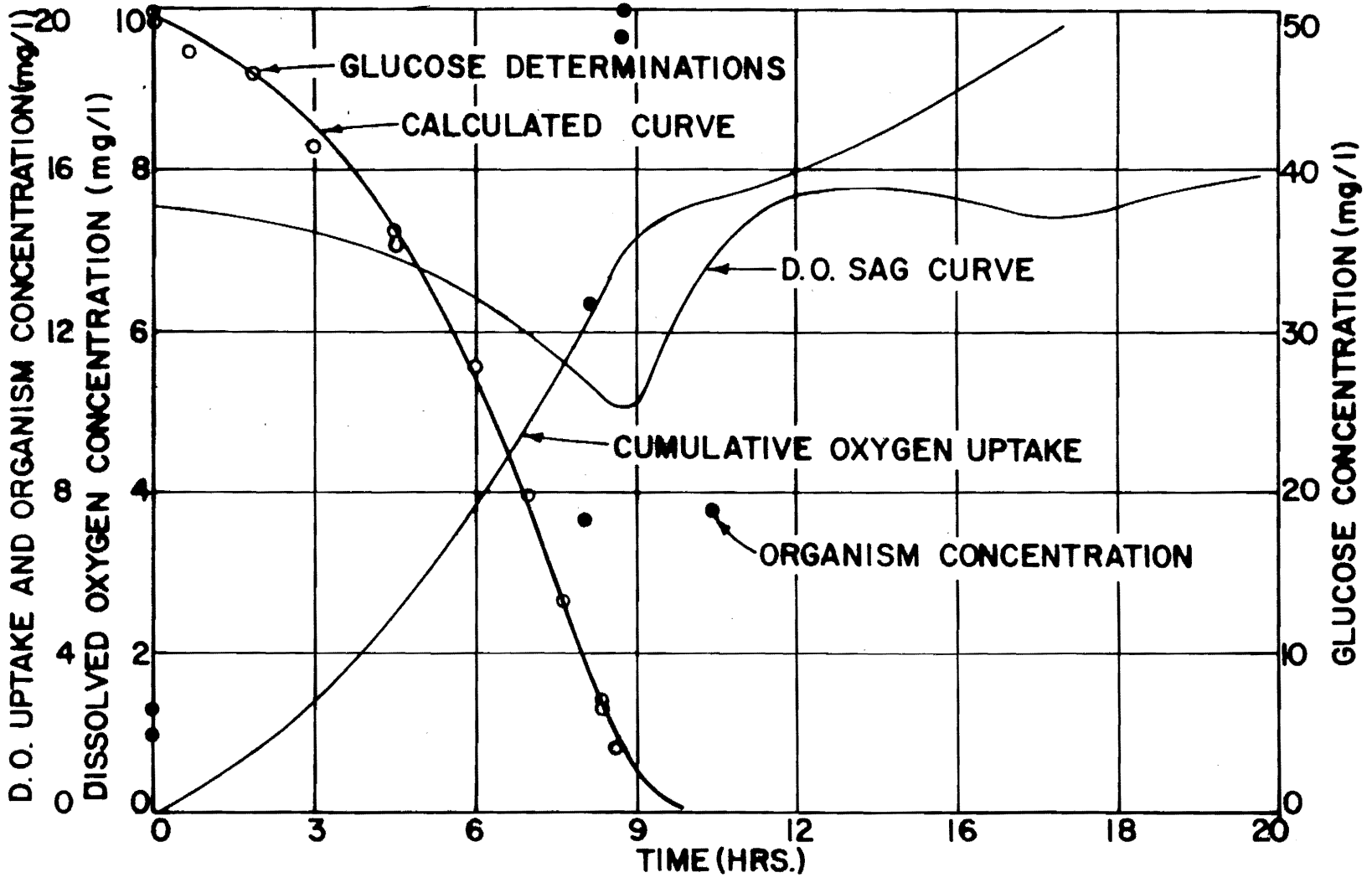


Figure 15. Oxygen Uptake, Glucose, D.O., and Organism Concentration as a Function of Time. Test No. 12

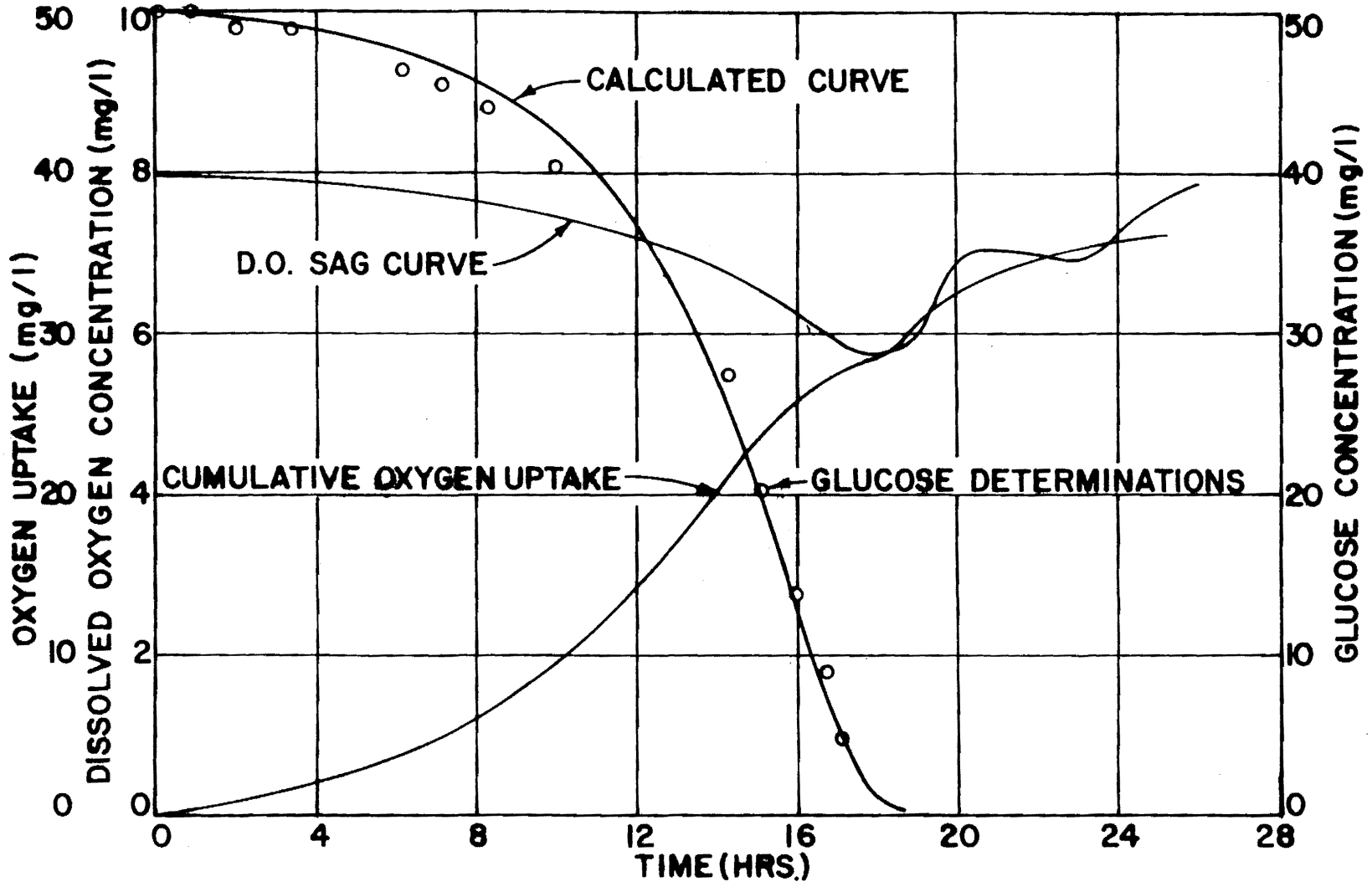


Figure 16. Glucose Concentration, Dissolved Oxygen Concentration and Oxygen Uptake as a Function of Time. Test No. 13

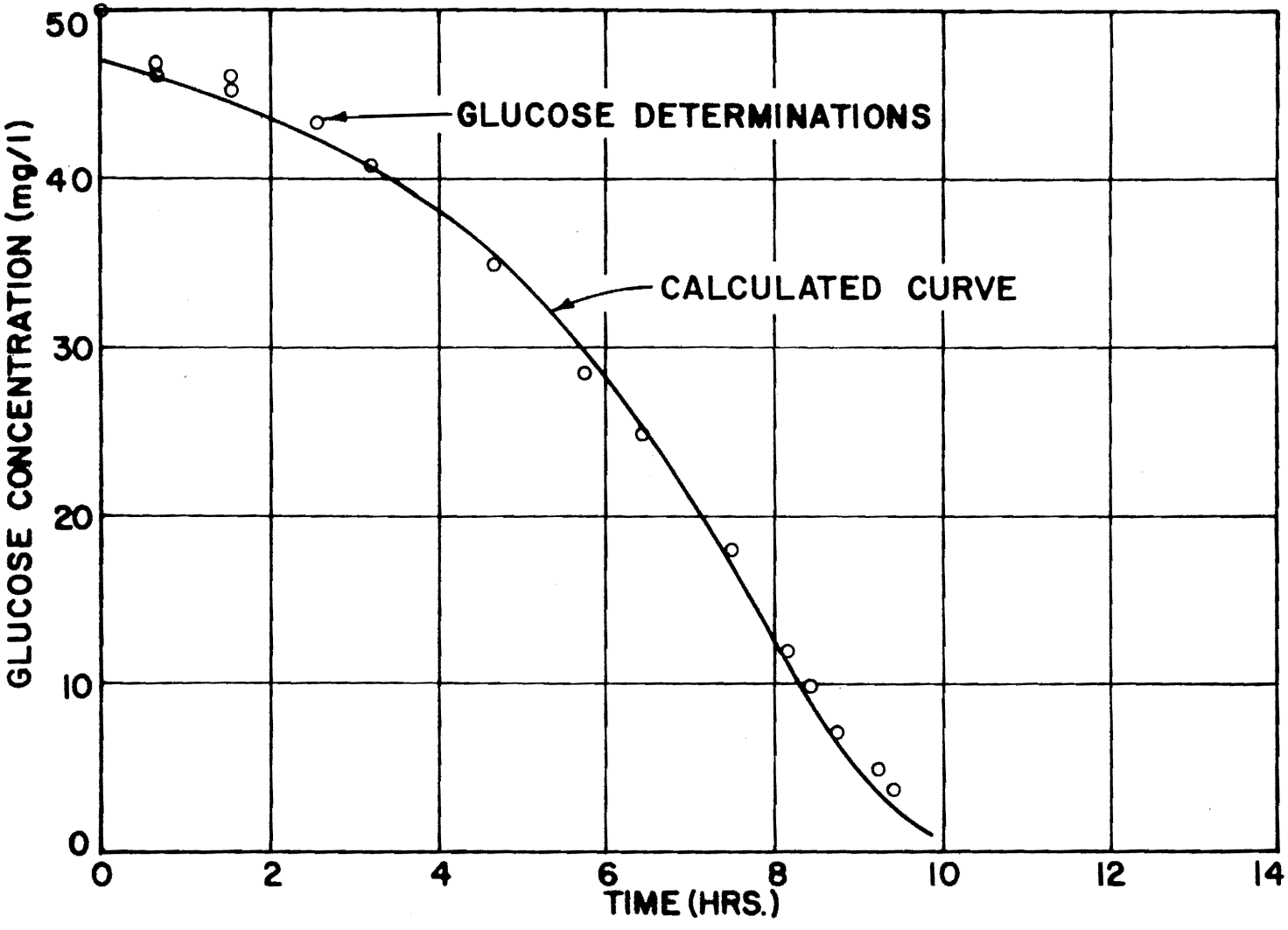


Figure 17. Glucose Concentration as a Function of Time. Test No. 14

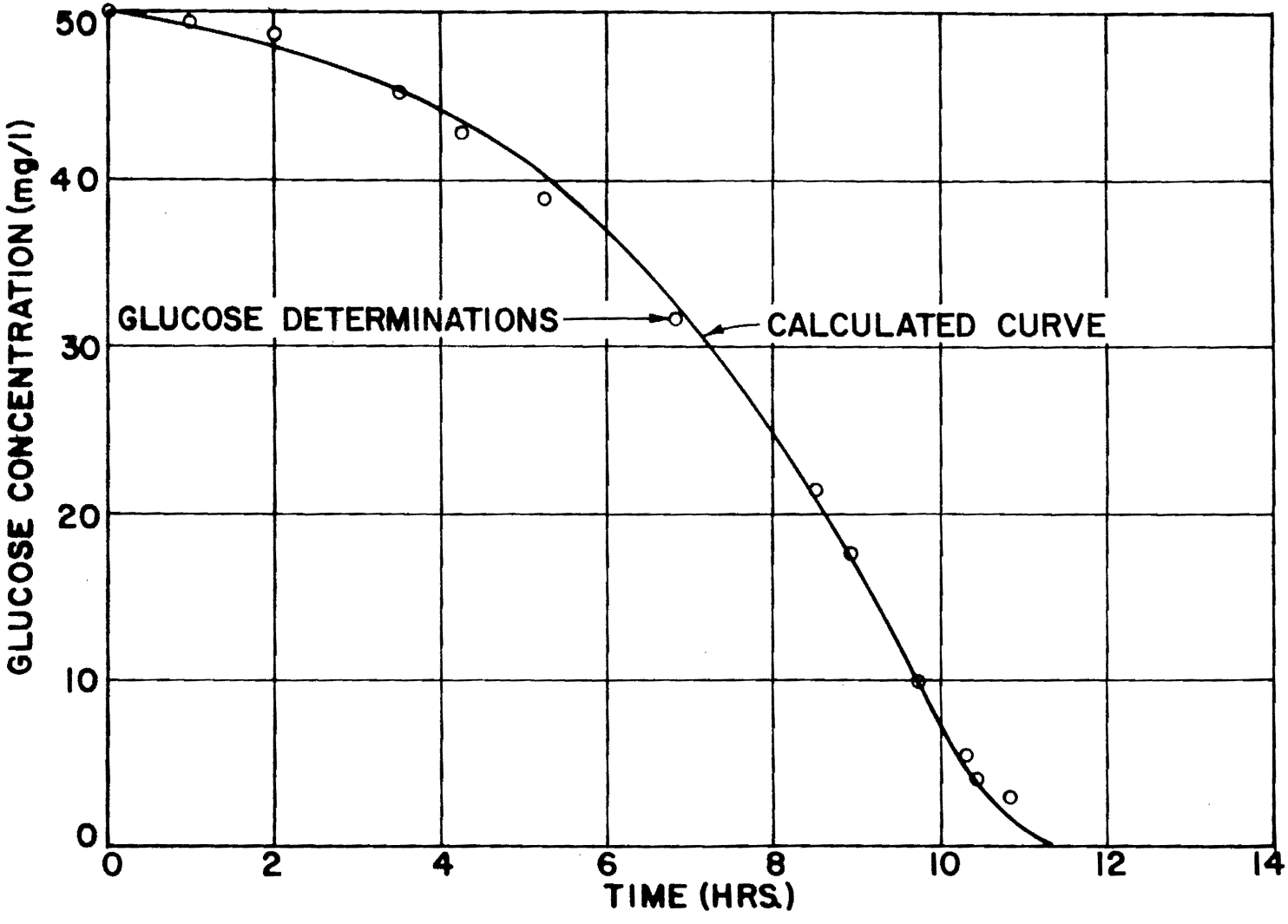


Figure 18. Glucose Concentration as a Function of Time. Test No. 15

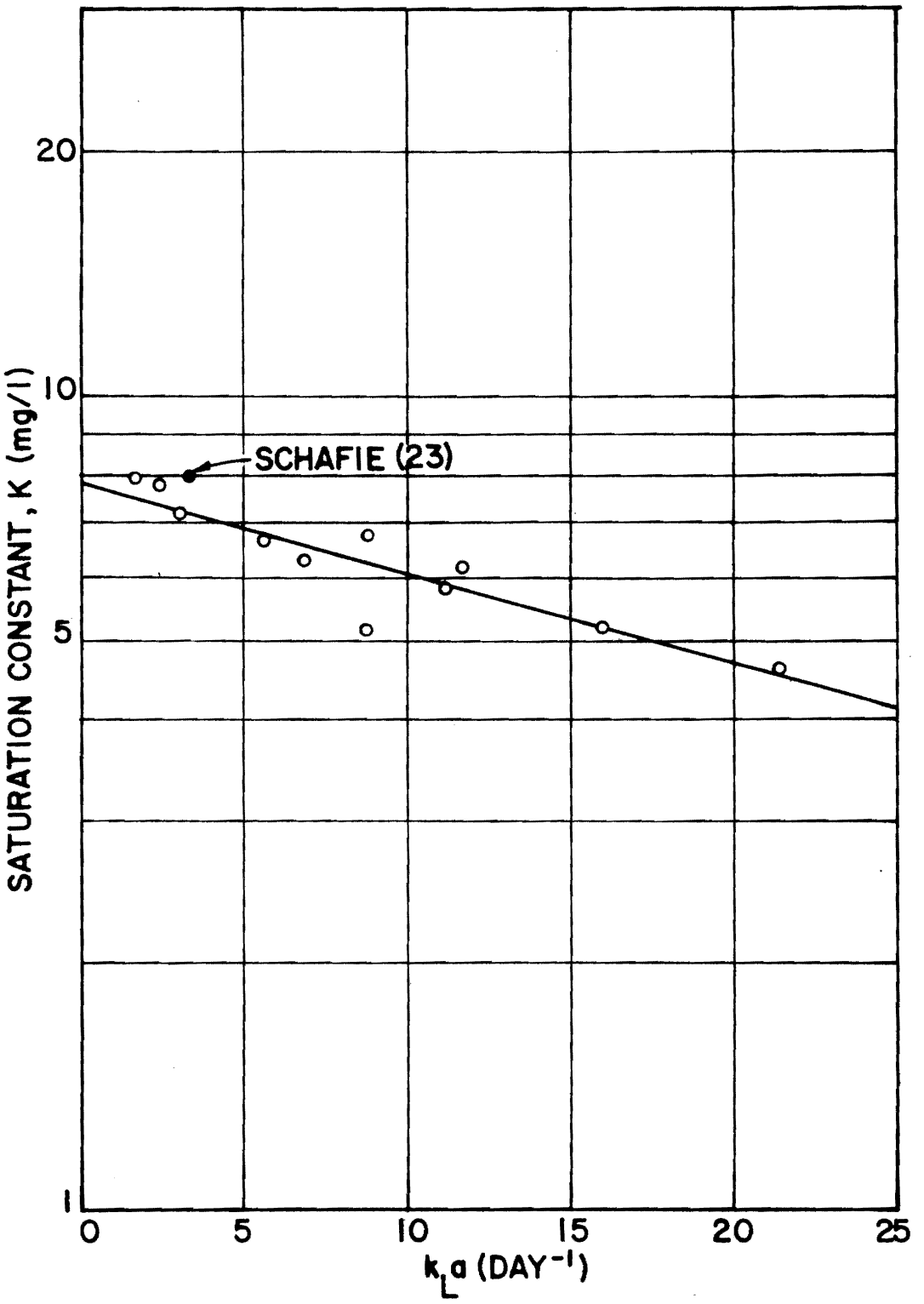


Figure 19. Saturation Constant, K, as a Function of Turbulence as Measured by $k_L a$

Discussion of Results

Evaluation of System Parameters

To adequately characterize the bacterial-substrate system investigated, it was necessary to evaluate each term in the Monod nutrient equation describing substrate utilization in order to separate variables from constants.

$$\frac{dx^n}{d\theta} = - \frac{\left[k^m X_\theta^n\right]}{\left[K + X_\theta^n\right]} \frac{\left[X_\theta^o\right]}{[Y^o]} \tag{9}$$

As seen in the differential form of the Monod equation, an important evaluation to be made was whether the maximum growth rate, k^m, and/or the substrate saturation constant, K, were constants or varied with environmental changes.

Since the organism concentration, X^o, and the bacterial yield, Y^o, are in a ratio, changes in the variable Y^o should not affect the other parameters or the response of the system to environmental changes. A variable yield would only affect the amount of oxygen utilized during bacterial growth. Although the objectives of this investigation do not require that Y^o remain constant, the work of other investigators presented earlier strongly suggest that it is, in fact, a constant. The accuracy of the values of Y^o determined in this investigation and presented in Table 3 depend entirely on the accuracy of the test used to determine the initial organism concentration because the bacterial yield was not measured directly, but is computed (10).

The values of Y^o in Table 3 are presented to demonstrate one potential use of the dehydrogenase test when combined with the approach to bacterial growth kinetics employed in this investigation.

Maximum growth rates, k^m, measured by other investigators were presented earlier in Table 2. Comparison of the values presented strongly suggests that k^m is a constant at a given temperature. It is believed that the metabolic pathway used by bacteria to utilize glucose is a more important consideration than the species of bacteria. Thus, the value of k^m is interpreted as being a true constant for aerobic bacterial systems using glucose as a substrate. The results obtained from the present investigation strongly support this concept. The range of the maximum growth rates computed from the data using the analytical techniques as presented by Gates and Marlar (10) are from 0.310 to 0.354 $hr.^{-1}$ with an average value of 0.336 hr^{-1}. Data presented by Schafie (23) using *E.coli* and glucose in a system similar to that used in the present investigation were analyzed and found to have a maximum growth rate of 0.340 hr^{-1}. The average k^m reported by other investigators as presented in Table 2 is 0.353 hr^{-1} which further substantiates the concept of a constant maximum bacterial growth rate under the conditions of this investigation.

No satisfactory or generally accepted definition of the substrate saturation constant, K, has been advanced in either the field of enzyme kinetics or microbiology. Riggs (61) stated that K_m, the Michaelis constant of enzyme substrate systems, is neither a rate constant nor a dissociation constant, but rather is a constant of "convenience." However, most other authors disagree with this view. Webb (62) points out

that K_m, can be either a "general Michaelis constant, a dissociation constant of the enzyme-substrate complex, or simply a kinetic constant" for enzyme-substrate systems depending upon the relative magnitude of the rates of enzyme-substrate complex formation and breakdown. These ideas are illustrated as follows:

$$\text{Enzyme} + \text{Substrate} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{Enzyme-Substrate Complex} \xrightarrow{k_3} \text{Enzyme} + \text{Products}$$

if $k_2 \simeq k_3$ then,

$$K_m = \frac{k_2 + k_3}{k_1} \quad ; \text{ a general Michaelis constant}$$

if $k_2 >> k_3$, then,

$$K_m = \frac{k_2}{k_1} \quad ; \text{ a dissociation constant of the ES complex}$$

if $k_2 << k_3$, then,

$$K_m = \frac{k_3}{k_1} \quad ; \text{ a kinetic constant}$$

Webb also stresses that the value of K can show the relative affinity of enzymes for different substrates. Karlson (63) adds that when the substrate concentration is numerically equal to the value of the Michaelis constant, half of the entire enzyme present is in the form of the enzyme-substrate complex and the other half is free enzyme. Also,

the value of the Michaelis constant is equivalent to the substrate concentration at which half-maximal reaction velocity is reached. This, however, is only a mathematical definition of the Michaelis constant in terms of the other parameters in the equation. Wilson (65) and other investigators in fields other than enzyme kinetics use the mathematical definition mentioned above as a working definition of the substrate saturation constant, K. It is apparent that no satisfactory definition of either the Michaelis constant, K_m, or the analogous substrate saturation constant, K, as applied to bacterial-substrate systems has been formulated.

Examination of Equation (9) in light of the previous discussion reveals that k^m and Y^o are constants. Since X^n and X^o are measurable parameters, any biochemical or environmental change in a bacterial-substrate system which influences the rate of substrate utilization will be reflected in the numerical value of K. No experimental data to evaluate this concept was found in the literature. Stratton and McCarty (19) presented data indicating that K had no dependence on temperature, while Knowles et al. (20) reported increasing K values with increasing temperatures. These two isolated investigations were the only works noted in which environmental changes of any kind were evaluated. While the influence of temperature was not considered in the present investigation, the above investigations are mentioned to illustrate the minimal amount of past work which has been done and the disagreement which exists. If the saturation constant does reflect all biochemical and environmental system changes, the Monod equations are even more desirable as a tool to gain insight into bacterial-substrate systems under a

variety of conditions.

Applicability of the Monod Equations

The substrate data plotted as a function of time presented in Figures 6 through 18 were analyzed using the analytical technique mentioned earlier (10). The obvious ability of the Monod nutrient equation to represent these data while meeting the requirements presented earlier of a constant maximum growth rate, k^m, and a system parameter, K, in which environmental changes are reflected is demonstrated. Changes in the value of K resulting from environmental changes will be discussed later. The ability to separate variables, constants, and measurable parameters in order to study the influence of selected variables makes the use of the Monod expressions particularly desirable.

The fact that varying initial organism concentrations greatly influences the resulting rate of substrate removal in bacterial-substrate systems has had limited acceptance in Sanitary Engineering. Figure 20 is a plot of curves calculated for data obtained from several tests in this investigation. This plot illustrates quite graphically the effect of the initial concentration of organisms. Also of significance is the ability of the Monod equation to adequately describe each test in terms of kinetic constants and measurable parameters. The data is completely characterized by the maximum bacterial growth rate, k^m, the substrate saturation constant, K, the bacterial yield, Y^o, and the initial organism concentration, X_o^o. Since k^m and Y^o can be considered constants and K is a constant for a given set of environmental conditions, the only remaining term in the Monod equation is the initial organism concentration, X_o^o. Using the techniques presented earlier,

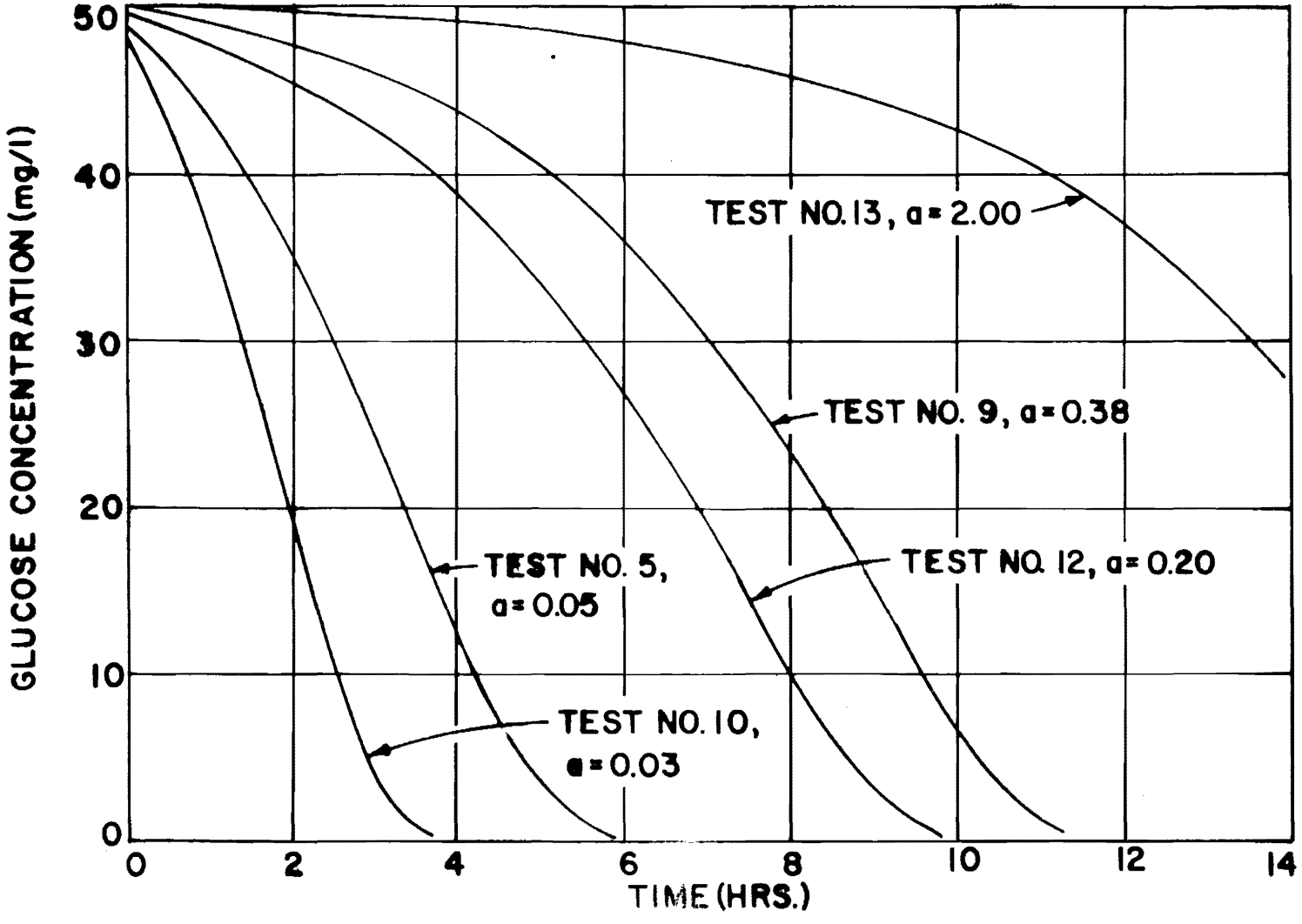


Figure 20. Glucose Concentration as a Function of Time Showing the Effect of Initial Organism Concentration

the viable initial organism concentration can be measured. Thus the effect of changes in the initial organism concentration can be predicted since organism concentration becomes a measurable parameter as opposed to an unknown variable, when the Monod expression is used. Without the use of this quantitative relationship between bacterial growth and the subsequent utilization of substrate, data such as presented in Figure 20 would have very little meaning other than to illustrate the effect of different initial organism concentrations.

Oxygen Uptake

Figure 10 shows the observed relationship between bacterial growth, substrate utilization and oxygen consumption. It is evident from these data that bacterial growth took place at the expense of the substrate with a concurrent utilization of oxygen shown as the cumulative oxygen uptake curve. The cumulative oxygen uptake curve was obtained by numerically integrating the D.O. sag curve. The curve obtained is the equivalent of a continuously monitored BOD test using a turbulent system rather than the traditionally quiescent system. The shape of the oxygen uptake curve was intimately related to the substrate utilization observed. In the early hours of the test, the slope of the oxygen uptake curve was seen to increase due to the rapidly increasing numbers of organisms and their subsequent substrate and oxygen utilization. As the substrate depleted to a low concentration, the D.O. concentration reached a minimum and began to rise. This was an indication of a reduced rate of oxygen consumption and was reflected in the cumulative oxygen uptake curve. The plateau of the oxygen uptake curve can be interpreted from these data as indicating the depletion of the

soluble substrate.

It should be noted that immediately following the oxygen plateau, another oxygen demand occurred. Bacterial activity, as measured by the dehydrogenase test, reached a peak corresponding closely to the depletion of the soluble substrate and then declined rapidly. In general, this second phase of oxygen uptake can be interpreted as the onset of autodestruction of the bacterial population. The term autodestruction should be differentiated from endogenous respiration. Endogenous respiration is a level of bacterial metabolism just sufficient to maintain the life functions of the bacteria. In the absence of exogenous energy sources, the bacterial population must rely on internal or endogenous energy stores. Once these have been exhausted, death and subsequent lysis of cell components into the bulk solution occurs. This action combined with the utilization of the released substances by the remaining live bacteria is termed autodestruction. This state is characterized by a temporary increase in the rate of oxygen uptake and an increasing total amount of oxygen utilized but at a decreasing rate. Some oxygen will continue to be utilized until the bacterial population depletes itself. The total amount of oxygen consumed during the sequence of growth and autodestruction is equivalent to the biochemical oxygen demand of the original substrate plus the biochemical oxygen demand of the initial organisms present.

Some investigators believe that endogenous respiration takes place at all times, even during the active growth of bacterial cultures. Others state that there is no evidence of endogenous respiration during active growth (65). The influence of endogenous respiration

during active growth, if it occurs, appears to be small enough to be neglected without serious error. The rates of "endogenous respiration" as given by Eckenfelder and O'Connor (27) range from 0.007 to 0.036 mg O_2/hr/mg of bacteria for specific species of bacteria and 0.00185 to 0.0098 mg O_2/hr/mg of sludge for activated sludges. Of the tests performed during the course of the present investigation, test number eight had the highest initial organism concentration, that is, approximately 13 mg/l, which, assuming no loss of organisms and a yield of 0.4 mg bacteria/mg glucose removed, results in a predicted increase of 18.8 mg/l of biomass. The maximum concentration of biomass present in the reactor would then be 31.8 mg/l which considering only endogenous respiration would utilize a maximum of:

(0.036 mg/l O_2/hr/mg/l bacteria)(31.8 mg/l bacteria) = 1.04 mg/l O_2/hr.

which is much lower than the observed 5 mg/l O_2/hr observed during the hour preceding the exhaustion of the soluble substrate. An attempt was made to correlate the oxygen utilized per milligram of glucose removed during active growth of the biomass with the initial organism concentration to test the hypothesis that endogenous respiration during active growth would be reflected in the total amount of oxygen utilized for depletion of the soluble substrate. No apparent correlation resulted. A better correlation was obtained between the cumulative oxygen uptake and $k_L a$, as shown in Figure 21. Although there appears to be a correlation between these two parameters, the correlation is likely to be spurious.

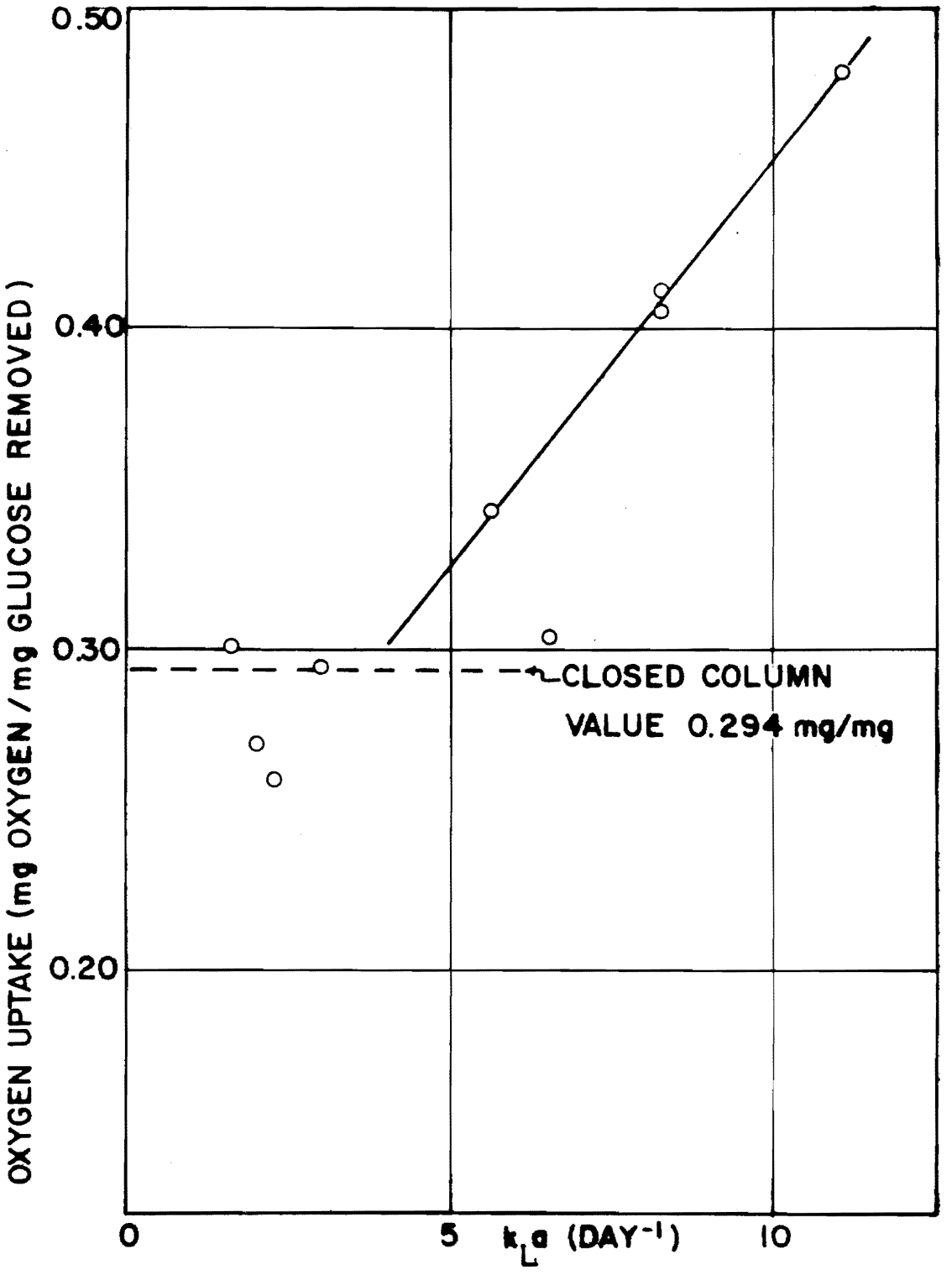


Figure 21. Ratio of Oxygen Used to Glucose Removed as a Function of Turbulence as Measured by $k_L a$

It is probable that the correlation obtained in Figure 21 represents cumulative errors resulting from numerical integration of the oxygen sag curve. Such errors of approximations would increase as the rate of reoxygenation increases. An additional measurement of oxygen utilization was obtained by measuring the cumulative oxygen consumption resulting from bacterial substrate utilization in a closed complete-mix system. The results of this test are presented in Table 3 and closely correspond to the data obtained from other tests involving low rates of reaeration. It is believed that oxygen utilization data obtained at the lower reaeration rates is more reliable for the reasons mentioned above.

Effect of Turbulence

The second objective of this investigation was to demonstrate the influence of turbulence on bacterial substrate utilization. Previous discussion has pointed out the suitability of the Monod equations to quantitatively measure the effect of environmental changes on bacterial-substrate systems.

Since the rate of reoxygenation is proportional to the degree of agitation imposed on the system, turbulence was measured by determining the overall rate of reoxygenation, $k_L a$. If the two-film theory (2) is applied conceptually to a complete-mix system, increased agitation causes a decrease in the thickness of a liquid film at the surface through which oxygen transfer from the atmosphere to the hydrosphere must take place. By decreasing the thickness of this liquid film, the resistance to molecular diffusion of oxygen is lowered, thus increasing the overall rate of oxygen transfer to the bulk solution. The transport

of substrate from the bulk solution to, or into, a bacteria is analogous to the transport of oxygen through a rate limiting resistance layer. It is probable that at some point the substrate in the bulk solution must be transported by molecular diffusion as it is utilized by the bacteria in dispersed growth systems. Dispersed growth is specified since substrate utilization by flocculent bacterial aggregates can become oxygen limited even when the concentration of oxygen in the bulk solution is non-limiting (14,66). Whether, in dispersed growth systems, the rate limiting step consists of diffusion through a laminar layer of water surrounding the bacterial cell induced by shear forces, a layer of "bound water," a slime layer surrounding the cell, the cell wall, or combinations of these has not been determined at this time. Presently, only changing the level of turbulence and determining the effect of the change on the observed bacterial substrate utilization is possible.

Figure 19 and Equation (8) represent the observed dependence of the substrate saturation constant, K, on the turbulence level as measured by $k_L a$. As the turbulence level increased, the value of K decreased in an exponential fashion. As shown in Equation (1), decreasing K increases the rate of substrate utilization. Figure 22 is a plot of the specific growth rate, k, as a function of substrate concentration for values of K taken from Figure 19 and illustrates the influence of turbulence induced changes of K on the specific growth rate. It is apparent that changes in the value of K have the greatest influence on the specific bacterial growth rate, k, at low substrate concentrations. The lower values of K have the effect of causing the specific bacterial growth rate to be higher at a given substrate concentration since the

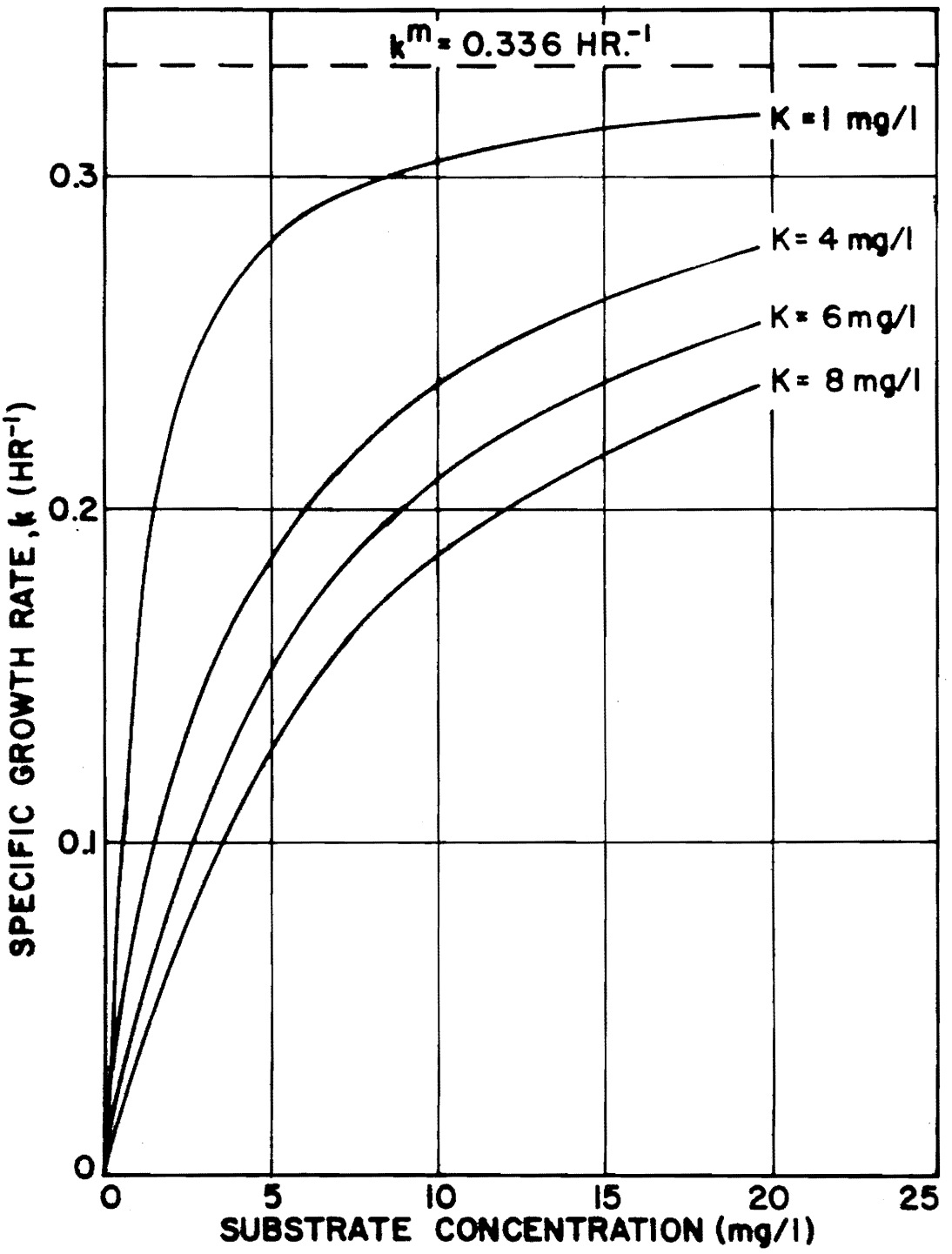


Figure 22. Specific Growth Rate, k, as a Function of Substrate Concentration

relation between k and K is:

$$k = \frac{k^m X_\theta^n}{K + X_\theta^n} \qquad (1)$$

Using an assumed initial organism concentration, Figure 23 further illustrates the effect that different values of K have on the substrate utilization curve. It is apparent that the substrate is utilized more rapidly when the value of the substrate saturation constant is low. In the case presented, the substrate in the turbulent system would be essentially depleted at 15 hours while more than 3 mg/l of substrate would remain in the quiescent system. The depletion of the substrate in the quiescent system would have taken an additional three hours or more which represents an increase of approximately 20 per cent.

As a point of comparison and a possible indication that measurements of turbulence by $k_L a$ may be compared for different systems, data presented by Schafie (23) were analyzed and are plotted in Figure 19. The value of K obtained was 8.0 mg/l and compares quite favorably with the data obtained during the present investigation.

Significance of Experimental Approach and Results

The "building block" experimental technique employed for this investigation is an approach which permits one to isolate the cause-effect relationship of selected environmental parameters on the rate of substrate utilization. In addition, as was the situation in this investigation, the range of variable, or variables, considered can be extended to include the anticipated values in a more complex or natural

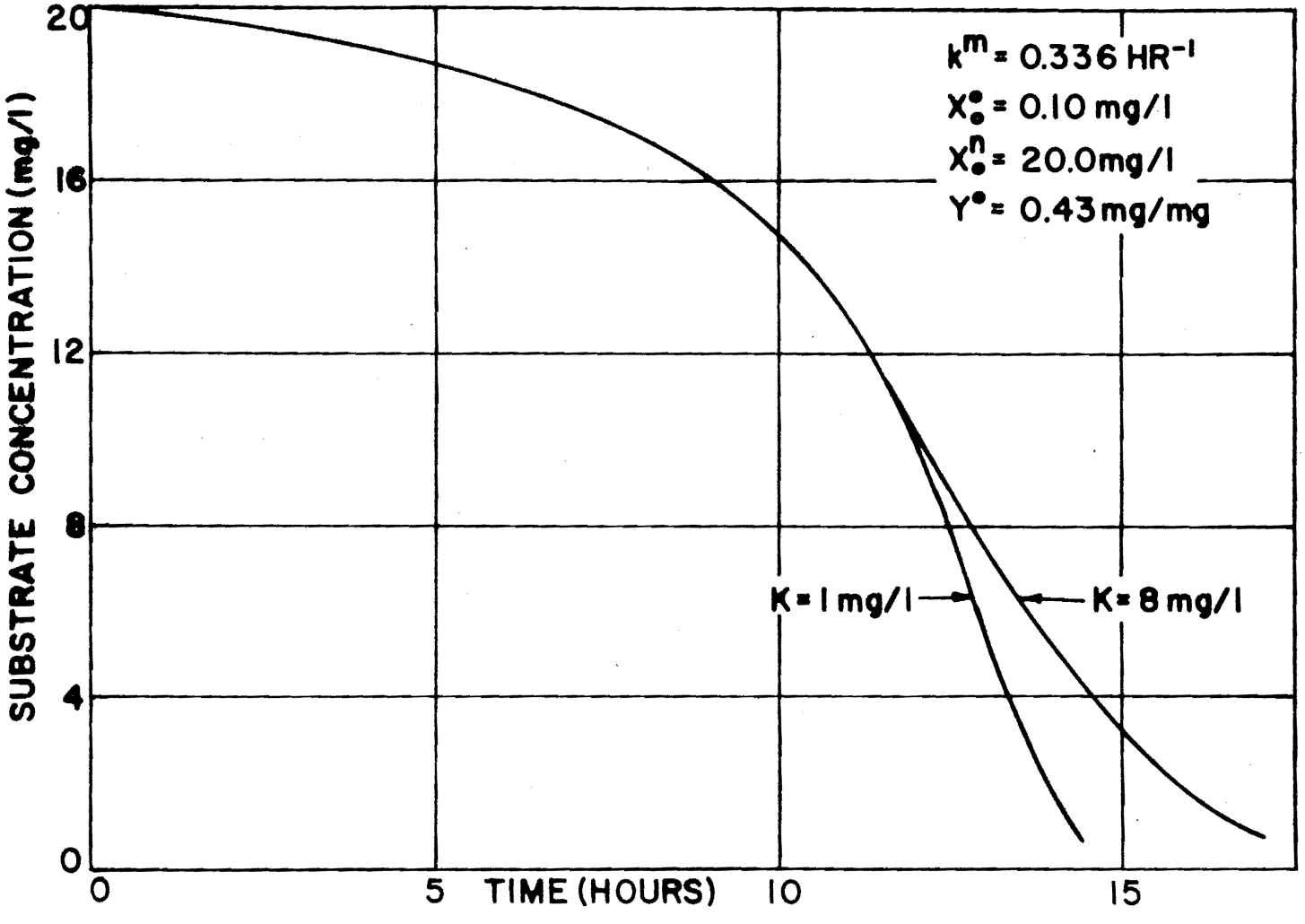


Figure 23. Substrate Concentration as a Function of Time for Different Values of the Saturation Constant, K

system. Therefore, in the more complex system the influence of the variable studied can be interpreted objectively rather than subjectively.

The results of this investigation represent a significant contribution to bettering the understanding of the interrelationships between bacterial substrate utilization and environmental parameters in general; and specifically the impact of organism concentration and turbulence on such substrate utilization.

The utility and applicability of the Monod model in the analysis of dilute heterogeneous culture heterogeneous culture systems have been adequately demonstrated. The capacity of the model to include the influence of organism concentration on the rate of substrate utilization in predicting this rate permits the inclusion of a significant environmental parameter in the analysis and prediction of both stream simulation and stream analysis studies.

The determination of the relationship between turbulence, as measured by $k_L a$, and the saturation constant demonstrates how the effects of secondary environmental factors may be manifested in both the model and the actual system. Thus, it appears probable that many other significant but not primary environmental parameters may exert influence on system and model behavior in this manner and should be evaluated in a similar fashion.

CHAPTER VII

CONCLUSIONS

From the results of this investigation it is concluded that for the system studied:

1. The bacterial growth kinetic approach presented can be utilized quite well with a heterogeneous bacterial population.

2. The effect of varying initial bacterial concentrations on the system is important and can be predicted.

3. Measurement of viable biomass in dilute systems is possible using a modified dehydrogenase test.

4. The oxygen utilization by the bacterial population is directly proportional to the utilization of substrate.

5. The utilization of substrate did not follow first order kinetics, but followed the form predicted by the mathematical model presented by Monod (1).

6. The maximum bacterial growth rate was a constant.

7. Environmental conditions affecting the specific growth rate of the bacteria and thus the rate of substrate removal were reflected in the value of the substrate saturation constant, K.

8. Increasing the level of turbulence in the bacterial-substrate system increased the specific bacterial growth rate and the rate of substrate removal, but not the maximum growth rate.

CHAPTER VIII

RECOMMENDATIONS

The results of this research have implications important to several aspects of sanitary engineering. Specifically, the experimental techniques and the mathematical model employed offer a mechanism by which a better understanding of the "self-purification" of streams can be achieved.

Since the Monod approach to bacterial growth kinetics appears to be suitable both in a practical and theoretical sense, the next logical step would be to identify and quantify as many variables as possible which affect bacterial growth and substrate utilization. Although such a task may be considered as basic research, economic, governmental, and social pressures are presently such that predictions of future stream conditions must be based upon the best predictor systems available. The classical analysis of the assimilative capacity of streams is being scrutinized both as to its engineering adequacy and theoretical basis.

Since the Monod approach describes the processes of bacterial growth and substrate utilization, the kinetic expressions used in this research should most precisely apply to situations in which bacterial *growth* occurs. Presently, it appears that streams receiving either raw domestic sewages, sewages receiving primary treatment, or any of a variety of soluble industrial wastes should be amenable to analysis

using the Monod expressions.

It is presently possible to utilize the Monod equations as "process models" to enable a reasonable analysis of existing stream conditions. It should be recognized that such a "process" approach to stream analysis will permit prediction of stream conditions under other environmental conditions, but any constants derived from the analysis of conventional field data, such as BOD data, will necessarily be in terms of the testing techniques employed. Thus, such constants should be recognized as being "process" constants rather than true kinetic constants. Since the evaluation of existing stream conditions and the predictions of stream behavior under other conditions is highly dependent upon the field and laboratory testing techniques employed, it is apparent that an evaluation of available techniques warrants serious consideration. Some of the available techniques which may be usable to measure the bacterial substrate are the BOD test, continuously monitored oxygen uptake studies, COD test, and the determination of organic carbon. It may be that one or more of these techniques will prove to be suitable for engineering purposes although it is probable that organic carbon analyses will prove to be the most suitable for both research and practical applications.

While it appears that the Monod approach to bacterial growth kinetics should be directly applicable to stream analysis, it has become apparent that much more laboratory research is needed. The separation and identification of a number of variables has yet to be accomplished. It is probable that the most expedient method to evaluate the effects of major variables affecting bacterial substrate utilization is through

the use of a simulation laboratory model, a heterogeneous bacterial population, and a well defined substrate. These same laboratory studies could be used to study the biochemical reactions occurring after bacterial growth has ceased. It is apparent that both the growth and death of bacteria are involved in the "self-purification" of streams. The identification, separation, and evaluation of one variable affecting bacterial growth has been demonstrated in the present research, but a precise delineation of the variables affecting the behavior of bacterial populations after the exhaustion of their substrate has yet to be made. Both are necessary if conditions existing in natural streams are to be evaluated and ultimately predicted.

In summary, the immediate application of bacterial growth kinetics to stream analysis should consist of:

1. laboratory research using a simplified system to identify, study, and evaluate the variables affecting bacterial substrate utilization,
2. an evaluation of existing testing techniques available to characterize complex wastes which are discharged to streams followed by laboratory studies to develop "process" constants for the particular waste, and
3. the application of the results obtained from laboratory studies to the analysis of streams subject to organic pollution.

Concurrent with the growth kinetic studies, an evaluation of variables affecting the behavior of bacterial populations after the exhaustion of available growth energy sources should be made.

It is the writer's opinion that meaningful analyses of polluted streams can be accomplished best through the use of laboratory simulation studies and the application of these results to situations existing in nature.

APPENDIX

APPENDIX 1

DERIVATION OF MONOD EQUATIONS

The equation used by Monod (1) to explain his experimental data was empirical in nature. Monod noted that a definite relationship existed between the concentration of substrate in a bacterial-substrate system and the bacterial growth rate. He then determined experimentally that an expression similar to that used by Michaelis and Menten to characterize enzyme-substrate systems could satisfactorily represent the relationship of the specific bacterial growth rate with a maximum growth rate, the substrate concentration and a substrate saturation constant. Even though Monod's initial use of this relationship was empirical, the same expressions can be derived by proposing a reaction scheme similar to that generally accepted for enzyme-substrate interactions. This concept will be demonstrated below.

Michaelis-Menten Expression

Michaelis and Menten proposed that enzyme-substrate reactions take place as follows:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P \qquad (9)$$

where: E = Enzyme

S = Substrate

ES = Enzyme-Substrate Complex

P = Reaction Products

In this form, the expression applies to steady state systems or those in dynamic equilibrium. In concentration units Equation (9) becomes:

$$X^E + X^S \underset{k_2}{\overset{k_1}{\rightleftarrows}} X^{ES} \xrightarrow{k_3} X^E + X^P \qquad (10)$$

Let: X^E = Total enzyme concentration in the system

X^S = Substrate concentration

X^{ES} = Enzyme-substrate Complex concentration

θ = Time

then $X^E - X^{ES}$ = Concentration of uncombined enzyme in the system

To obtain an expression for the rate at which substrate is converted to product the following considerations are necessary.

The rate of complex (ES) formation is:

$$\frac{dX^{ES}}{d\theta} = k_1(X^E - X^{ES})(X^S) \qquad (11)$$

The rate of complex (ES) breakdown is:

$$\frac{-dX^{ES}}{d\theta} = k_2X^{ES} + k_3X^{ES} \qquad (12)$$

When the system is in dynamic equilibrium, the rate of complex formation is equal to the rate of complex breakdown, thus from Equations (11) and (12):

$$k_1(X^E - X^{ES})(X^S) = k_2X^{ES} + k_3X^{ES} \tag{13}$$

Simplifying Equation (13) results in:

$$\frac{k_2 + k_3}{k_1} = \frac{(X^E - X^{ES})(X^S)}{X^{ES}} \tag{14}$$

Equation (14) is the definition of K_m, the Michaelis-Menten Constant, that is:

$$K_m = \frac{k_2 + k_3}{k_1} \tag{15}$$

Using Equation (14) the value of the concentration of enzyme-substrate complex is:

$$X^{ES} = \frac{X^E X^S}{K_m + X^S} \tag{16}$$

The rate of conversion of substrate to product is:

$$\frac{dX^S}{d\theta} = k_3X^{ES} = V \tag{17}$$

Where: V = the velocity of the reaction.

When the concentration of substrate (X^S) is very high, the concentration of uncombined enzyme will be essentially zero and the rate of conversion of substrate to product will be at a maximum, thus:

$$X^E - X^{ES} = 0$$

or
$$X^E = X^{ES} \tag{18}$$

Also, the maximum reaction velocity at which substrate can be converted to product is:

$$V_{max} = k_3 X^E \tag{19}$$

Solving Equation (17) and substituting into Equation (16) yields:

$$V = k_3 \left[\frac{X^E X^S}{K_m + X^S} \right] \tag{20}$$

Substituting Equation (19) into Equation (20) gives:

$$V = \frac{V_{max} X^S}{K_m + X^S} \tag{21}$$

Equation (21) is the Michaelis-Menten equation relating the velocity of conversion of substrate to product to the maximum velocity at which conversion can take place, the substrate concentratin at any time, and the Michaelis-Menten Constant.

An Expression for Bacterial-Substrate Systems

The similarity between Equations (1) and (21) may be accidental or may indicate that a similar line of reasoning may be used to arrive

at the basic expressions first used by Monod (1). Consider the following reaction schemes:

$$\text{Bacteria} + \text{Substrate} + \text{Oxygen} \rightarrow \text{Bacteria} + \text{Products}$$

or

$$\text{Bacteria} + \text{Substrate} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{Bacteria-Substrate} \xrightarrow{k_3} \text{Bacteria} + \text{Products}$$

Let: X^o = Bacterial Concentration

X^n = Substrate Concentration

X^{on} = Bacterial-Substrate Complex Concentration

$X^o - X^{on}$ = Bacteria not involved in complex

θ = Time

Assuming, initially, that the concentration of bacteria remains constant, the rate of complex formation is:

$$\frac{dX^{on}}{d\theta} = k_1(X^o - X^{on})X^n \tag{22}$$

The rate of complex breakdown is:

$$\frac{-dX^{on}}{d\theta} = k_3(X^{on}) + k_2(X^{on}) \tag{23}$$

At a steady state situation:

$$-\frac{dX^{on}}{d\theta} = \frac{dX^{on}}{d\theta}$$

Thus, equating Equations (22) and (23) yields:

$$\frac{k_2 + k_3}{k_1} = \frac{(X^o - X^{on})\, X^n}{X^{on}} = K \tag{24}$$

Then let:

$$K = \frac{k_2 + k_3}{k_1} \tag{25}$$

Equation (25) is often used as the definition of the substrate saturation constant in the Monod expressions.

The rate of substrate conversion to product is:

$$-\frac{dX^n}{d\theta} = k_3 X^{on} = V \tag{26}$$

Then, as before, V is the velocity of the reaction and if X^n is sufficiently high such that all the bacteria are involved in the complex, then:

$$X^o - X^{on} = 0$$

and

$$X^o = X^{on}$$

Then Equation (26) becomes:

$$\frac{-dX^n}{d\theta} = k_3 X^o = V_{max} \tag{27}$$

Substituting Equations (26) and (27) into Equations (24) yields:

$$V = \frac{V_{max} X^n}{K + X^n} \tag{28}$$

It should be noted that Equation (28) is identical to Equation (21). In bacterial-substrate systems, however, it is obvious that an increase in the numbers of bacteria result from conversion of the substrate to its products. When bacteria are actively growing in an exponential manner their rate of growth may be expressed as:

$$\frac{dX^o_\theta}{d\theta} = k\ X^o_\theta \tag{29}$$

where: k = Specific growth rate.

However, it can also be stated that:

$$\frac{dX^o_\theta}{d\theta} = Y^o \left[\frac{dX^n_\theta}{d\theta}\right] \tag{30}$$

where: Y^o = Bacterial Yield = $\frac{\text{mg. organisms produced}}{\text{mg. substrate removed}}$.

However, from Equation (26):

$$V = \frac{-dX^n_\theta}{d\theta}$$

Therefore:

$$\frac{dX_{\theta}^{o}}{d\theta} = Y^{o}V \tag{31}$$

Equating Equations (29) and (31) yields:

$$kX_{\theta}^{o} = Y^{o}V$$

or

$$V = k\left[\frac{X_{\theta}^{o}}{Y^{o}}\right] \tag{32}$$

And also:

$$V_{max} = \frac{k^{m}X_{\theta}^{o}}{Y^{o}} \tag{33}$$

Substitution of Equations (32) and (33) into Equation (28) results in:

$$k = \frac{k^{m}X_{\theta}^{n}}{K + X_{\theta}^{n}} \tag{1}$$

Equation (1) is the basic Monod expression for the bacterial growth rate at any time. The Monod expression for substrate concentration in batch culture systems as used in this investigation is developed as follows.

$$\frac{dX^n}{d\theta} = -\left[\frac{1}{Y^o}\right] \frac{dX^o_\theta}{d\theta} \tag{30}$$

But:

$$\frac{dX^o_\theta}{d\theta} = kX^o_o \tag{29}$$

And:

$$k = \frac{k^m X^n_\theta}{K + X^n_\theta} \tag{1}$$

Therefore:

$$\frac{dX^n_\theta}{d\theta} = -\left[\frac{1}{Y^o}\right]\left[\frac{k^m X^n_\theta}{K + X^n_\theta}\right] X^o_\theta \tag{34}$$

In order to integrate Equation (34), it is necessary to get the organism concentration, X^o_θ, in terms of the substrate. Thus:

$$Y^o(X^n_o - X^n_\theta) = (X^o_\theta - X^o_o)$$

or

$$X^o_\theta = X^o_o + Y^o(X^n_o - X^n_\theta) \tag{35}$$

Substituting Equation (35) into Equation (34):

$$\frac{dX_\theta^n}{d\theta} = -\left[\frac{1}{Y^o}\right]\left[\frac{k^m X_\theta^n}{K + X_\theta^n}\right] [X_o^o + Y^o(X_o^n - X^n)] \tag{36}$$

Rearrangement of Equation (36) results in:

$$-\left[\frac{k^m}{Y^o}\right] d\theta = \frac{K\, dX_\theta^n}{X_\theta^n(X_o^o + Y^o X_o^n - Y^o X_\theta^n)} + \frac{dX_\theta^n}{(X_o^o + Y^o X_o^n - Y^o X_\theta^n)} \tag{37}$$

Integrating:

$$-\left[\frac{k^m}{Y^o}\right] \int_o^\theta d\theta = K \int_{X_o^n}^{X_\theta^n} \frac{dX_\theta^n}{X_\theta^n(X_o^o + Y^o X_o^n - Y^o X_\theta^n} + \int_{X_o^n}^{X_\theta^n} \frac{dX_\theta^n}{(X_o^o + Y^o X_o^n - Y^o X_\theta^n)}$$

Resulting in, after rearrangement:

$$\ell n(X_\theta^n) = \ell n\left[[X_o^o + Y^o(X_o^n - X_\theta^n)]\left[\frac{X_o^n}{X_o^o}\right]\right] + \left[\frac{X_o^o + Y^o X_o^n}{Y^o K}\right] \ell n\left[\frac{X_o^o + Y^o(X_o^n - X_\theta^n)}{X_1^o}\right]$$

$$- \frac{k^m \theta (X_o^o + Y^o X_o^n)}{Y^o K} \tag{38}$$

Equation (38) is the Monod expression for the substrate concentration at any time in a batch system.

APPENDIX 2

ADDITIONAL REAGENTS

Saline Phosphate Buffered Solution

Dissolve 1.182 gm of KH_2PO_4, 5.61 gm of K_2HPO_4, and 14.8 gm of NaCL in one liter of distilled water. This solution was used to wash the substrate from centrifically harvested bacteria to be used as seed organisms or for other purposes.

Nutrients Added to Test Reactor

	Item	Stock Solution	Amount Used
1.	Na_2SO_4	73.40 gm/l	Use 5 ml/l
2.	$FeCl_3 \cdot 6H_2O$	0.40 gm/l	5 ml/l
3.	$(NH_4)_2SO_4$	44.13 gm/l	5 ml/l
4.	KH_2PO_4	10.24 gm/l	
	& K_2HPO_4	13.11 gm/l	5 ml/l
5.	$CaCl_2 \cdot 2H_2O$	0.40 gm/l	5 ml/l
6.	$MgCl_2 \cdot 6H_2O$	32.00 gm/l	5 ml/l

APPENDIX 3

EXPERIMENTAL DATA

Test 5

Time (Hours)	Glucose Concentration (mg/l)	Organism Concentration (mg/l)
0	50.0	10.0,* 9.1*
0.87	43.3, 42.8	
2.42	29.8, 30.3	
3.25	20.5	
3.78	13.0	
4.27	9.0	
4.93	3.4, 3.0	

Test 6

0	50.0, 48.0, 48.7	7.6,* 6.4*
0.75	43.2, 43.3	
1.67	36.0, 39.5	
2.48	31.2, 32.0	
2.92	28.1	
3.42	24.0, 23.0	
3.92	19.9	
4.15		6.0, 4.5
4.42	13.0, 13.7	
4.92	8.7	
5.17	5.7	25.6, 20.8
5.70	2.5, 1.8	
5.97	2.0, 0.8	
6.05		35.6, 33.7
7.20		35.6, 6.7

---

*Values calculated from dehydrogenase tests performed on bacterial "seed" solutions before the "seed" was added to the reactor.

Test 7

Time (Hours)	Glucose Concentration (mg/l)	Organism Concentration (mg/l)
0	19.4	6.2,* 1.9,* 4.4*
0.15	19.1	
1.10	16.0	
1.28		2.1, 3.5, 3.5
1.87	13.4	
2.55	11.7	
3.03		6.7, 9.0, 9.8
3.14	9.0, 9.2	
3.64	7.2, 7.1	
4.20	4.5, 4.6	
4.58		17.8, 15.5
4.72	3.0, 3.8	
5.08	1.4	
5.32		14.9, 8.0
6.62		8.8, 10.0

Test 8

0		
0.27	47.0, 47.0	
0.77	45.2, 45.2	
1.27	44.0, 44.0	
2.27	42.0	
3.00	39.0, 39.5	
3.83	37.4, 36.8	
4.83	32.8, 30.5	
6.90	13.0	
7.38	9.8, 9.0	
7.63	6.8	
8.08	2.8	

Test 9

0	50.0, 50.0	0.9,* 0.78,* 1.19*
0.70	49.8, 49.8	
1.55	47.0, 48.5	
2.42	45.8, 45.8	
4.00	44.2, 43.1	
4.34		2.8, 2.9
6.00	36.8, 36.5	
8.00	23.0, 22.7	
9.00	16.5, 16.5	
9.63	10.8, 11.2	

	Time (Hours)	Glucose Concentration (mg/l)	Organism Concentration (mg/l)
Test 9 Continued	10.50	4.7, 4.0	
	10.77	2.5	
		Test 10	
	0	50.0, 48.4	
	0.28	45.8, 45.8	
	0.63	41.0, 41.4	
	1.10	35.1, 35.3	
	1.67	23.9, 23.5	
	2.32	11.2, 11.2	
	2.72	5.8, 6.8	
	3.17	1.4	
		Test 11	
	0	46.8, 47.8	2.96,* 4.22*
	0.67	44.0, 45.1	
	1.60	40.4, 38.9	
	2.62	31.8, 32.4	
	3.50	26.1, 26.1	
	4.32	18.2, 19.5	
	5.00	13.1, 12.8	
	5.50	8.3, 8.3	
	5.78	4.1	
	6.10	2.5	
		Test 12	
	0	49.8, 49.3	2.6,* 2.0,* 2.1*
	0.67	47.2, 47.2	
	1.73	46.0, 46.0	
	3.00	41.5, 41.4	
	4.50	36.3, 35.5	
	6.00	27.8, 27.8	
	7.00	19.8, 19.2	
	7.67	13.2, 13.2	
	7.72		7.3, 13.7
	8.33	6.9	
	8.67	4.0	20.0, 19.3
	9.93		7.6

Time (Hours)	Glucose Concentration (mg/l)	Organism Concentration (mg/l)
	Test 13	
0	50.0	0.3,* 0.3*
0.18	48.8	
0.83	50.0, 50.0	
2.00	48.8	
3.35	48.8	
6.15	46.4	
7.17	45.4	
8.25	44.0	
10.00	40.4	
12.00	36.0	
14.22	27.5	8.4, 7.7
14.80		
15.13	20.3	
16.00	19.9	
16.72	8.9	
17.17	4.8	
17.23		
	Test 14	
0	50.0, 50.0	
0.65	47.0, 46.3	
1.58	45.9, 46.3	
2.55	43.5	
3.18	42.0	
4.68	35.0	
5.75	28.5	
6.45	25.0	
7.50	18.0	
8.17	12.0	
8.48	10.0	
8.75	7.3	
9.25	5.0	
9.43	3.8	

Time (Hours)	Glucose Concentration (mg/l)	Organism Concentration (mg/l)
	Test 15	
0	50	
1.0	49.5	
2.0	48.7	
3.5	45.2	
4.28	42.9	
5.25	38.8	
6.83	31.6	
8.50	21.4	
8.92	17.7	
9.75	10.0	
10.33	5.5	
10.47	4.0	
10.83	3.0	

REFERENCES CITED

1. Monod, J., "Recherches sur la Croissance des Cultures, Bacteriennes." Hermann et Cie, Eds. Paris, France (1942).

2. Haney, P. D., "Theoretical Principles of Aeration," *Journal American Water Works Association, 46,* 4, 353 (April 1954).

3. Streeter, H. W. and Phelps, E. B., *Public Health Bulletin,* 146, U. S. Public Health Service, Washington (1925).

4. Fair, G. M., "Water Pollution Research and the Revolution in Science and Technology," *Advances in Water Pollution Research,* Proceedings of the International Conference held in London, September, 1962. Pergamon Press, Ltd., *1*, XI (1964).

5. Kittrell, F. W., and Kochtizky, O. W., "Natural Purification Characteristics of a Shallow Turbulent Stream," *Sewage Works Journal, 19,* 6, 1032 (June 1947).

6. Velz, C. J. and Gannon, J. J., "Biological Extraction and Accumulation in Stream Self Purification," In *Advances in Water Pollution Research.* Proceedings of the International Conference held in London, September 1962. Pergamon Press Ltd., Vol. 1, 1 (1964).

7. Schroepter, G. J., Robins, M. L., and Susag, R. H., "The Research Program on the Mississippi River in the Vicinity of Minneapolis-St. Paul," in *Advances in Water Pollution Research.* Proceedings of the International Conference held in London, September 1962. Pergamon Press Ltd., Vol. 1, 137 (1964).

8. Mancy, K. H., and Gates, W. E., "Application of a Laboratory Model to Study Assimilative Capacities of Streams," Presented at the Symposium on the Capacity of Streams to Assimilate Wastes, American Chemical Society, Division of Water, Air, and Waste Chemistry, 152nd Meeting, New York City (September 1966).

9. Gates, W. E., Pohland, F. G., Mancy, K. H., and Schafie, F. R., "A Simplified Model for Studying Assimilative Capacity," *Proceedings of the 21st Industrial Waste Conference,* Purdue University Extension Service, *121*, 665 (1966).

10. Gates, W. E., and Marlar, J. T., "Graphical Analysis of Batch Culture Data Using the Monod Expressions," Submitted to *Journal Water Pollution Control Federation* (August 1967).

11. Monod, J., "The Growth of Bacterial Cultures," *Annual Review of Microbiology,* Vol. 3, 371 (1949).

12. Karlson, P., *Introduction to Modern Biochemistry,* Academic Press New York and London, Second Edition (1965).

13. Webb, J. L., *Enzyme and Metabolic Inhibitors,* Academic Press, New York, and London, Vol. 1 (1963).

14. Downing, A. L., and Wheatland, A. B., "Fundamental Considerations in Biological Treatment of Effluents," *Transactions of the Institution of Chemical Engineers. Vol. 40,* 2, 91 (1962).

15. Gaudy, A. F., Jr., Ramanathan, M., and Rao, B. S., "Kinetic Behavior of Heterogeneous Populations in Completely Mixed Reactors," *Biotechnology and Bioengineering, 9,* 3, 387 (1967).

16. Grieves, R. B., Milbury, W. F., and Pipes, W. O., Jr., "A Mixing Model for Activated Sludge," *Journal Water Pollution Control Federation, 35,* 5, 619 (1964).

17. Andrews, J. F., and Willimon, E. P., Jr., "Multi-Stage Biological Processes for Waste Treatment," Presented at the 22nd Annual Purdue Industrial Waste Conference, Purdue University (May 1967).

18. Malek, I., and Fencl, Z., *Theoretical and Methodological Basis of Continuous Culture of Microorganisms.* Academic Press, N. Y. and London (1966).

19. Stratton, F. E., and McCarty, P. L., "Prediction of Nitrification Effects on the Dissolved Oxygen Balance of Streams," *Environmental Science and Technology, 1,* 5, 405 (May 1967).

20. Knowles, G., Downing, A. L., and Barrett, M. J., *Journal of General Microbiology,* Vol. 38, pp. 263-278 (1965).

21. Fair, G. M., and Geger, J. C., *Water Supply and Waste-Water Disposal,* John Wiley and Sons, Inc. (1954).

22. Rich, L. G., *Unit Processes of Sanitary Engineering,* John Wiley and Sons, Inc. (1963).

23. Schafie, F. R., "A Simplified Model to Study Natural Purification in Streams," M.S. Thesis, University of Cincinnati (1965).

24. Isaacs, W. P., and Gaudy, A. F., Jr. "Comparison of BOD Exertion in a Simulated Stream and in Standard BOD Bottles," Presented at the 22nd Industrial Waste Conference, Purdue University (May 1967).

25. Busch, A. W., Grady, L., Jr., Rao, S. T., and Swilley, E. L., "Short-Term Total Oxygen Demand Test," *Journal Water Pollution Control Federation, 34,* 4, 354 (April 1962).

26. Butterfield, C. T., Purdy, W. C., and Theriault, E. J., "Experimental Studies of Natural Purification in Polluted Waters--IV, The Influence of Plankton on the Biochemical Oxidation of Organic Matter," *Public Health Reports, 46,* 393 (1931).

27. Eckenfelder, W. W., and O'Conner, D. J., *Biological Waste Treatment,* Pergamon Press (1961).

28. Agardy, E. J., Cole, R. D., and Pearson, E. A., "Enzyme Activity as a Parameter of Digester Performance." *Proceedings of the 18th Industrial Waste Conference,* Purdue University, Extension Service *115*, 51 (1963).

29. Lenhard, G., Nourse, L. D., and Schwartz, H. M., "The Measurement of Dehydrogenase Activity of Activated Sludges," Section II, Paper No. 6, *Second International Conference on Water Pollution Research,* Tokyo, Japan (1964), Pergamon Press (1964).

30. Ford, D. L., and Eckenfelder, W. W., "The Effect of Process Variables on Sludge Floc Formation and Settling Characteristics," *Technical Report to the U. S. Public Health Service,* EHE-11-6604 (1966).

31. Hernandez, J. W., "Aerobic Bacterial Uptake of Dissolved Nutrients Between 10° and 70° C.," Ph.D. Thesis, Harvard University (1965).

32. Pipes, W. O., and Micholits, E. M., "Aerobic Yield and Theoretical Oxygen Demand," *Proceedings of the 18th Industrial Waste Conference,* Purdue University Extension Service, *115,* 418 (1963).

33. Gaudy, A. F., Jr., and Gaudy, E. T., "Microbiology of Waste Waters," *Annual Review of Microbiology, 20,* 319 (1966).

34. Watson, J. A., and Hoffee, P. A., "The Glucose Effect and the Relationship between Glucose Permease, Acid Phosphate, and Glucose resistance," Cold Spring Harbor Symposia on Quantitative Biology, *26,* 261 (1961).

35. Morris, J. G., *Journal of General Microbiology, 22,* 564 (1960).

36. Servizi, J. A. and Bogan, R. H., "Free Energy as a Parameter in Biological Treatment," *Journal of the Sanitary Engineering Division, ASCE,* 69, SA 3, 17 (June 1963).

37. Englebrecht, R. S. and McKinney, R. E., "Activated Sludge Cultures Developed on Pure Organic Compounds," *Sewage and Industrial Wastes, 29,* 12, 1350 (December 1957).

38. McKinney, R. E., and Symons, J. E., *Advances in Water Pollution Research,* Proceedings of the International Conference held in London, September, 1962, Pergamon Press, Ltd., *2*, 440 (1964).

39. Neidhardt, F. C., and Frankel, D. G., "Metabolic Regulation of RNA Synthesis in Bacteria," Cold Spring Harbor Symposia on Quantitative Biology, *26,* 63 (1961).

40. Moyed, H. S., "Interference with Feedback Control of Enzyme Activity," Cold Spring Harbor Symposia on Quantitative Biology, *26*, 323 (1961).

41. Bush, A. W., and Myrick, R., "Aerobic Bacterial Degradation of Glucose," *Journal Water Pollution Control Federation, 33,* 897 (September 1961).

42. Gaudy, A. F., Jr., Bhatla, M. N., Follett, R. H., and Abu-Niaaj, F., "Factors Affecting the Existence of the Plateau During the Exertion of BOD," *Journal Water Pollution Control Federation, 37,* 4, 444 (April 1965).

43. McWhorter, T. R., and Heukelekian, H., "Growth and Endogenous Phases in the Oxidation of Glucose," *Advances in Water Pollution Research,* Proceedings of the International Conference held in London, September, 1962, Pergamon Press, Ltd., *2*, 419 (1964).

44. Prokesovia, Vera, "Influence of Aeration on BOD," *Journal Water Pollution Control Federation, 34,* 12, 1268 (December 1962).

45. Nejedly, A. and Pelz, I., "Studies on the Longitudinal Mixing in Open Channels and its Effect on the Rate of the BOD Reaction in Aqueous Media, "Hydraulic Research Institute Prague-Podbaba, Czech. (1964).

46. Bhatla, M. N., and Gaudy, A. F., Jr., "Studies on the Causation of Phasic Oxygen Uptake in High Energy Systems," *Proceedings of the 19th Industrial Waste Conference,* Purdue University Extension Service, *117,* 871 (1964).

47. Dobbins, W. E., "BOD and Oxygen Relationships in Streams," *Journal of the Sanitary Division,* ASCE, *90,* SA3, 53 (June 1964).

48. Sanders, W. M., III, "The Growth and Development of Attached Stream Bacteria, Part I. Theoretical Growth Kinetics of Attached Stream Bacteria," *Water Resources Research, 3,* 1, 81 (1967).

49. Kehrberger, G. J., Norman, J. D., Schroeder, E. D., and Busch, A. W., "BOD Progression in Soluble Substrates--VII Temperature Effects," *Proceedings of the 19th Industrial Waste Conference,* Purdue University Extension Service *117,* 953 (1964).

50. Symons, G. E., Discussion of "Natural Purification Characteristics of a Shallow Turbulent Stream," by Kittrell and Kochtizky, *Sewage Works Journal, 19,* 6, 1048 (June 1947).

51. Swilley, E. L., Bryant, J. O., and Busch, A. W., "Significance of Transport Phenomena in Biological Oxidation Processes," *Proceedings of the 19th Industrial Waste Conference,* Purdue University Extension Service *117,* 821 (1964).

52. Hartman, L., "Influence of Turbulence on the Activity of Bacterial Slimes," *Journal Water Pollution Control Federation, 39,* 6, 958 (June 1967).

53. Kittrell, F. W., and Furfari, S. A., "Observations of Coliform Bacteria in Streams," *Journal Water Pollution Control Federation, 35,* 11, 1361 (November 1963).

54. Frost, W. H. and Streeter, H. W., *Public Health Bulletin 143,* U.S. Public Health Service, Washington (1924).

55. Mancy, K. H., and Westgarth, W. C., "A Galvanic Cell Oxygen Analyzer," *Journal Water Pollution Control Federation, 34,* 1037, 1962.

56. Mancy, K. H., and Jaffe, T., "Analysis of Dissolved Oxygen in Natural and Waste Waters," *Public Health Service Publication No. 999-WP-37* (1966).

57. Martin, H. H., "Bacterial Protoplasts--A Review," *Journal of Theoretical Biology,* 15, 1 (1963).

58. Malalyan, M. N., and Biryuzova, V. I., "Localization of Dehydrogenase Activity in Mitochondrial Analogs of Bacterial Cells," *Proceedings of the Academy of Sciences of the U.S.S.R.,* Biological Sciences Section, 160 (January-February 1965).

59. Tengerdy, R. P., Nagy, J. G., and Martin, B., "Quantitative Measurement of Bacterial Growth by the Reduction of Tetrazolium Salts," *Applied Microbiology, 15,* 4, 954 (July 1967).

60. *Standard Methods for the Examination of Water and Waste Water, 12th Edition,* American Public Health Association, Inc., New York, 1965.

61. Riggs, D. S., *The Mathematical Approach to Physiological Problems,* The Williams and Wilkins Company (1963).

62. Webb, J. L., *Enzyme and Metabolic Inhibitors*, Vol. 1, Academic Press, New York and London (1963).

63. Karlson, P., *Introduction to Modern Biochemistry*, Academic Press, New York, and London (1965).

64. Wilson, I. S., "Concentration Effects in the Biological Oxidation of Trade Wastes," *Journal of Air and Water Pollution*, *77*, 99 (1963).

65. Mallette, M. F., "Validity of the Concept of Energy of Maintenance," Annals of the New York Academy of Science, *102*, 3 (1963).

66. Mueller, J. A., Boyle, W. C., and Lightfoot, E. N., "Oxygen Diffusion Through a Pure Culture Floc of Zooglea Remigera," *Proceedings of the 21st Industrial Waste Conference*, Purdue University Extension Service, *121*, 964 (1966).